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From Antibodies to Diagnosis: FOXP3 and PD-1 Markers of Human Lymphoid Malignancies

Doctoral Thesis

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CERTIFICA:

Que doña Giovanna Roncador ha realizado el presente trabajo: "From Antibodies to Diagnosis: FOXP3 and PD-1 Markers of Human Lymphoid Malignancies" que a mi juicio reune plenamente todos los requisitos necesarios para optar al **grado de Doctor,** a cuyo efecto será presentado en la Universidad Autónoma de Madrid. El trabajo se ha realizado bajo mi dirección, autorizando su presentación ante el tribunal correspondiente.

Y para que conste se extiende el presente certificado

Madrid a 5 de Noviembre de 2012

VºBº del director de la Tesis

Dr. Miguel Angel Piris

To Professor David Mason

We miss your insatiable curiosity, your acute observations, and your cheeky school-boy smile but your spirit live on with us.

There are two possible outcomes: if the result confirms the hypothesis, then you've made a measurement. If the result is contrary to the hypothesis, then you've made a discovery.

Enrico Fermi

ABSTRACT/RESUMEN

Abstract

Hybridoma technology, developed in the mid-1970s by Köhler and Milstein, made it possible to produce limitless quantities of highly specific monoclonal antibodies (mAbs) that recognize specific epitopes of cellular antigens. This advance has led to invaluable insights into normal lymphocyte differentiation and has permitted the identification of the cellular origins of several lymphoid malignancies. Improved immunohistochemical techniques, using a wide variety of antibodies in combination with morphology and molecular studies, have not only facilitated diagnosis and dramatically improved the classification of lymphoid malignancies but also provide an additional tool to predict tumour response to therapy.

In recent times, many studies have highlighted the rule of two helper T cell subtypes, regulatory T cells (T_{reg}) and follicular helper T cells (T_{FH}) as key players in tumour development and immunity. Both cell types have a variety of functions in T cell activation and in the maintenance of immune homeostasis, and they have been shown to play an important role in the development of autoimmune diseases as well as in cancer. Understanding the mechanisms by which T_{reg} and T_{FH} cells exert their influence in cancer is an area of intense research with broad implications for the development of therapeutic strategies. However, due to the lack of T_{reg} and T_{FH} specific markers, little was known about the presence or distribution of T_{reg} and T_{FH} in normal and tumoral tissues.

To address these issues we have produced and characterized several monoclonal antibodies against the transcription factor FOXP3. We have identified and documented for the first time the expression of FOXP3 protein at single cell level in both lymphoid tissue and peripheral blood, showing that FOXP3 mAb is a specific and sensitive toll able to detect FOXP3 protein in a variety of commonly used immunological techniques. Because of the importance of FOXP3 in the development and function of T_{reg} cells, we have analysed FOXP3 protein expression in 172 paraffin-embedded lymphoma samples. We found that FOXP3 in tumour cells was confined to adult T-cell leukaemia/lymphoma (ATLL) cases (17 out of 25 cases, 68%), while in the other lymphoma types analysed, FOXP3 expression was only detected in the reactive T cell population present in the tumour microenvironment. In a collaborative study we also

reported the presence of T_{reg} in the lymphoid microenvironment. In contrast with most studies done in solid tumours, we found that an increased number of T_{reg} is associated with prolonged survival in Hodgkin and follicular lymphomas, suggesting that FOXP3+ cells may act differently in this type of malignancies.

In an effort to better define the expression and the diagnostic value of two T_{FH} proteins, PD-1 and SAP, we have generated a specific mAb able to recognize PD-1 protein in paraffin embedded tissue and we have evaluated its expression in a large set of reactive and tumoral samples.

The immunohistochemical analysis of more than 500 lymphoma biopsies showed that 95% of the cases of angioimmunoblastic T-cell lymphoma (AITL) expressed at least one of these markers, providing additional evidence that AITL arises from T_{FH}, and they are likely to be a valuable tool in the diagnosis of this disease. PD-1 and SAP were also expressed in a minority of cases of peripheral T cell lymphoma other than AITL, raising the possibility that such cases may be related in origin to germinal center helper T cells.

We also reported that PD-1 is an excellent marker for the identification of the characteristic T-cell rosettes around the neoplastic B cells in nodular lymphocyte-predominant Hodgkin lymphoma (NPHL). We describe for the first time that the presence of PD-1 positive rosettes is not a unique feauture of NPHL, since we also found them in several cases of lymphocyte-rich classical Hodgkin lymphoma (LRCHL), suggesting a close biological relationship between these pathological entities. The presence of PD-1+ T-cell rosettes could be useful for the differential diagnosis of NLPHL and T cell /histiocyte-rich B cell lymphoma (THRBCL), which is usually a controversial and difficult task.

In conclusion, the screening of antibodies in routine sections has once again proven to be a powerful approach for identifying diagnostic biomarkers.

FOXP3 and PD-1 mAbs may help diagnostic pathologists in the identification of neoplastic T cells in routinely processed tissue samples and may also be used to achieve a better understanding of the pathogenic role of T_{reg} and T_{FH} cells in inflammatory and malignant diseases.

Resumen

La técnica para la producción de hibridomas, desarrollada en la década de los 70 por Köhler y Milstein, ha permitido generar cantidades ilimitadas de anticuerpos monoclonales (mAbs) capaces de reconocer con alta afinidad epítopos específicos de diferentes antígenos celulares. Gracias a este gran avance tecnológico, ha sido posible esclarecer tanto el proceso de diferenciación de los linfocitos normales como la identificación del origen celular de la mayoría de las neoplasias linfoides. La mejora de las técnicas inmunohistoquímicas junto con el uso de un amplio panel de anticuerpos, en combinación con los estudios morfológicos y moleculares, han facilitado el diagnóstico, revolucionando la clasificación de los linfomas y aportado una valiosa herramienta de apoyo pronóstico. Estudios recientes han destacado el papel fundamental realizado por dos subtipos de células T colaboradoras, las T reguladoras (T_{reg}) y las T colaboradoras del centro germinal (T_{FH}), como actores principales tanto en la inmunidad como en el desarrollo de tumores. Ambos tipos celulares intervienen en la regulación de la activación de células T y en el mantenimiento de la homeostasis inmunológica. Recientemente se ha puesto de manifiesto la implicación directa de estos tipos celulares en el desarrollo tanto en enfermedades autoinmunes, así como en cáncer. La elucidación de los mecanismos por los que las células T_{reg} y T_{FH} actúan en diferentes neoplasias es un área de investigación con una gran proyección en el desarrollo de nuevas estrategias terapéuticas. Sin embargo, la carencia de marcadores específicos frente a las poblaciones linfoides T_{reg} y T_{FH} dificultaba la comprensión sobre su presencia y distribución en tejido normal y tumoral.

Dado que las técnicas inmunohistoquímicas son una potente herramienta para poder abordar estos problemas, hemos producido y caracterizado varios anticuerpos monoclonales frente al factor de transcripción FOXP3. Por primera vez hemos identificado y documentado la expresión de FOXP3 a nivel celular, tanto en el tejido linfoide como en sangre periférica, demostrando que al anticuerpo FOXP3 constituye un erramienta específica y sensible capaz de detectar la proteína con una gran variedad de técnicas inmunológicas de rutina. Debido a la importancia que FOXP3 tiene en el desarrollo y función de las células T_{reg}, hemos estudiado su expresión en 172 muestras de linfoma fijadas y parafinadas. En tejido tumoral, su expresión se

encuentra restringida a la leucemia/linfoma de células T del adulto (ATLL) (17 de 25 casos, un 68%). Sin embargo, en el resto de los tumores estudiados, la expresión de FOXP3 fue detectada en la población T reactiva presente en el microambiente tumoral. Este resultado fue corroborado en un estudio en colaboración con otros laboratorios, donde se confirmó la presencia de T_{reg} en el microambiente linfoide. Sorprendentemente, a diferencia de la mayoría de los estudios realizados en tumores sólidos, se encontró que una mayor presencia de linfocitos T_{reg} se asociaba a una mayor supervivencia en linfomas de Hodgkin y folicular, lo que sugiere que la las celulas FOXP3 actuan de manera diferentes en estos tipos de linfomas.

Para poder definir con mayor precisión el patrón de expresión y el posible valor diagnóstico de las proteínas de PD-1 y SAP, marcadores especificosde la T_{FH}, hemos generado un mAb específico capaz de reconocer la proteína PD-1 en tejido fijado y parafinado. Se ha evaluado su expresión en una extensa serie de casos tanto reactivos (no tumoral) como tumorales. El estudio inmunohistoquímico de más de 500 biopsias de linfoma reveló que el 95% de los casos de linfoma T angioinmublástico (AITL) expresaban al menos uno de estos marcadores, dato que refuerza la hipótesis de que los AITL derivan de las células T_{FH}, por lo que estos nuevos marcadores constituyen una valiosa herramienta diagnóstica para esta enfermedad. La expresión de PD-1 y SAP también se encontró en una minoría de casos de linfoma T periférico, sugiriendo la posibilidad de que estos casos se originen en las células T_{FH} de centro germinal. Otro hallazgo fundamental ha sido la identificación de PD-1 como un excelente marcador de las rosetas de linfocitos T que rodean las céulas B neoplásicas, característica clave en el diagnóstico del linfoma de Hodgkin de predominio linfocítico (NPHL). Por primera vez se ha descrito que la presencia de rosetas PD-1+ no es una característica exclusiva del NPHL, sino que también se puede encontrar en un porcentaje elevado de linfomas de Hodgkin rico en linfocitos (LHCRL), lo que sugiere una estrecha relación biológica entre estas entidades. La presencia de rosetas PD-1+ constituye además una ayuda adicional en el difícil diagnóstico diferencial entre LHPLN y linfoma B rico en células T/histiocitos (THRBCL), dado que hemos encontrado que todos los casos analizados de THRBCL carecen de rosetas PD1+.

En conclusión, el uso de mAbs en el diagnóstico histopatológico de rutina ha demostrado una vez más ser una potente herramienta para la identificación de biomarcadores diagnósticos.

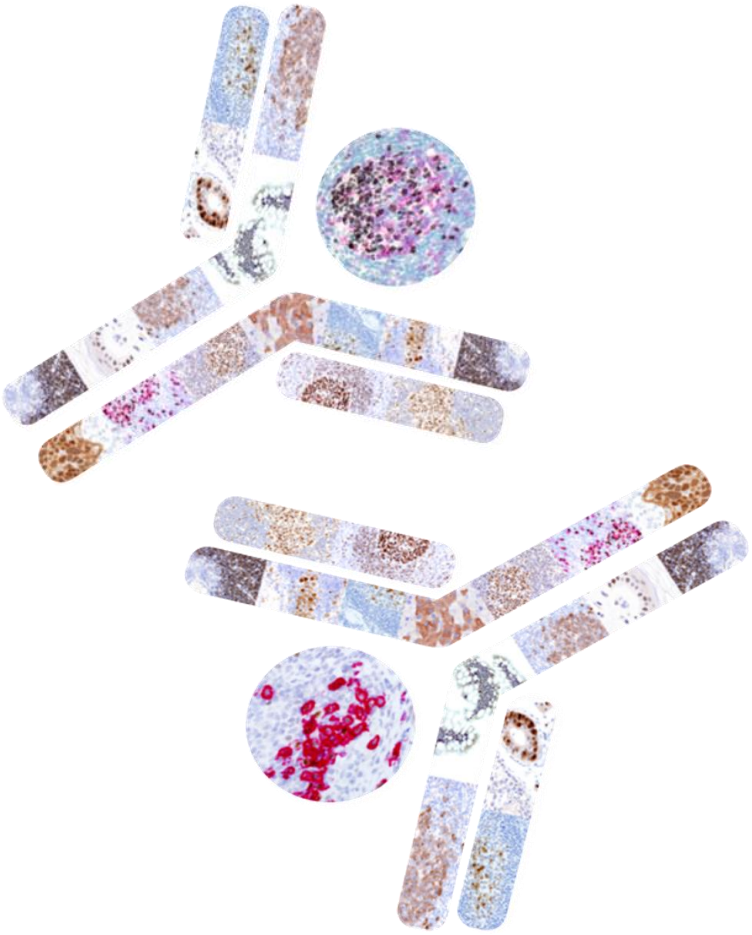
Los anticuerpos monoclonales FOXP3 y PD-1 han resultado ser unos excelentes marcadores para la identificación de las células T_{reg} y T_{FH} tanto en condiciones fisiológicas como patológicas, permitiendo un mejor conocimento del papel que estos linfocitos juegan tanto en procesos inflamatorios como en las enfermedades linfoproliferativas.

ABBREVATIONS

Abbreviations

AITL	Angioimmunoblastic T-cell lymphoma
APCs	Antigen-presenting cells
BCL6	B-cell lymphoma 6
CXCR5	C-C chemokine receptor type 5
CCR4	C-C chemokine receptor type 4
CCR7	Chemokine (C-C motif) receptor 7
CCL22	Chemokine (C-C motif) ligand 22
CD25	Interleukin 2 receptor α –chain
CD39	Ectonucleoside triphosphate diphosphohydrolase-1
CD73	Ecto-5´-nucleotidase
CHL	Classical Hodgkin's lymphomas
CSTCL	Cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma
CTLA-4	Cytotoxic associated antigen-4
DAB	3,3'-Diaminobenzidine
DCs	Dentritic cells
ELISA	Enzyme-linked immuno-absorbant assay
FCS	Fetal calf serum
FFPE	Formalin-fixed paraffin embedded
FOXP3	Forkhead box protein P3
FL	Follicular lymphomas
FR4	Folate receptor 4
GC	Germinal center
GEP	Gene expressio
GITR	Glucocorticoid-induced TNF receptor n profiling
HAT	Hypoxanthine, aminopterin and tymidine
H&E	Hematoxylin and eosin
ICOS	T-cell co-stimulator
IDO	Enzyme indoleamine 2,3-dioxygenase
IL-10	Interleukin 10
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-2R	Interleukin-2 receptor
IL-35	Interleukin 35
LRCHL	Lymphocytes reach classical Hodgkin lymphoma
IFN-γ	Interferon-γ
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iT_{reg}	Inducible regulatory T cell
MAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MHC II	Major histocompatibility complex class II
MCHL	Mixed cellularity classical Hodgkin lymphoma
NK	Natural killer
NLPHL	Nodular lymphocyte-predominant Hodgkin lymphoma
NSHL	Nodular sclerosis Hodgkin lymphoma
nT_{reg}	Naturally occurring regulatory T cells
OMS	Organización Mundial de la Salud
PBS	Phosphate-buffered saline
PD-1	Programmed cell death 1
PD-L1	Programmed death ligand 1

PD-L2 Programmed death ligand 2
PTCLs Peripheral T cell lymphomas
SAP Signalling lymphocytic activation molecule (SLAM)-associated protein
SLAMF SLAM family members
SDS-PAGE Sodium dodecyl sulfate–polyacrylamide gel
TCR T cell receptor
TGF-β Transforming growth factor beta
T_{FH} Follicular helper T cell
T_{eff} T effectors cells
T_H Helper T cell also called effector T cells
T_{H1} T helper 1
T_{H2} T helper 2
T_{H17} T helper 17
THRBCL T cell /histiocyte-rich B cell lymphoma
TMA Tissue microarrays
T_{reg} Regulatory T cell
XLP Immunodeficiency X-linked lymphoproliferative disease
WHO World Health Organization



INTRODUCTION

1. T cell lymphomas: Seeking for new biomarkers

The lymphoid malignancies are a heterogeneous group of disorders that occur as a result of neoplastic transformation of B and T lymphocytes at different stages of B- and T-cell differentiation. The wide variety of lymphoid malignancies reflects the various stages of lymphocyte development and the complexity of the immune system. The clinical and pathological characteristics of the lymphoid malignancies are collected in the World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues (WHO Classification) that uses all available information (morphology, cytochemistry, immunophenotype, genetics, and clinical features) to define clinically significant lymphoid entities (1). The WHO classification categorized lymphomas into three groups: B-cell neoplasms, T- and natural killer (NK) cell neoplasms and Hodgkin disease.

In contrast to the major progress made in the knowledge and classification of B-cell lymphomas, advances in the field of T-cell lymphomas have been much slower, and dealing with this category of diseases currently remains a challenge from the therapeutical and pathobiological perspectives.

Malignancies derived from mature T and NK cells (also called peripheral T cell lymphomas PTCLs), constitute a heterogeneous group of uncommon neoplasias, accounting about the 15% of all non-Hodgkin lymphomas worldwide. With few exceptions, PTCLs exhibit resistance to standard chemotherapy regimens and have a poor clinical outcome (2).

The histopathological diagnosis of T cell lymphomas is often challenging due to a broad morphologic and immunophenotypic variability and the significant overlaps across distinct T cell entities (2).

Recently, gene expression profiling (GEP) of this type of tumours has provided novel insights into the pathobiology of T cell lymphoma and led to the identification of novel biomarkers with diagnostic, prognostic or therapeutic implications (3, 4). In detailed, a recent GEP study has been crucial in establishing molecular similarities between one of the most common PTCLs, angioimmunoblastic T-cell lymphoma (AITL), and a subset of CD4+ T cell, normally located in germinal centre (follicular helper T cells (T_{FH})) that exert helper functions to follicular B cells (5).

In addition, several reports have highlighted the importance of another subset of helper T cells, called regulatory T (T_{reg}) cells, in lymphomagenesis. T_{reg} has recently become the focus of interest, due to the critical role of these cells in the modulation of immune responses, particularly in the suppression of tumor-associated antigen-reactive lymphocytes and consequently in cancer immunity (6).

The identification and the production of specific markers able to identify specific T cells lineage could be extremely useful to better understand the role that T cells subtypes, such as T_{reg} or T_{FH}, play in tumour development and in tumour microenvironment.

The ability to diagnose and classify lymphoid malignancies improved substantially in the 1980s thanks to the work of Professor David Mason and others (7) that developed an immunopathological method utilizing a wide variety of monoclonal antibodies (mAbs) against lymphoid cell surface antigens. This novel methodology was adapted to routine formalin-fixed paraffin embedded (FFPE) sections making a dramatic impact in pathology (8).

This technology was rapidly adopted by pathologists both at academic centres and in hospitals and is currently used, in conjunction with morphology and molecular studies, for the classification and diagnosis of lymphoid malignancies.

The aim of this thesis is the production of new cell lineage and differentiation markers specific for T cells (T_{reg} and T_{FH}). These mAbs will help to reveal the tumour's biological behaviour and will allow the detection of this cell types in paraffin tissue samples. This new markers may also help to refine the diagnostic and prognostic stratification of the patients with T cell lymphomas.

2. Helper T cells (T_H)

CD4+ T cells represent one of the main components of the adaptive immune response. These cells control the functional activities of both innate and adaptive immunity and determine the outcome of the immune response against infections. These cells do not have any cytotoxic activity *per se* and do not kill infected cells or clear pathogens directly. They instead control the immune response by directing other cells to perform these tasks.

Naïve T cells enter secondary lymphoid organs in the T cell zones and after the interaction with antigen-presenting cells (APCs), they can differentiate into subsets of

helper (effectors) CD4+ T cells (T_H) (T_{H1}, T_{H2}, T_{H17}, and T_{reg}) (9). Each subset is defined and controlled by a unique master regulator transcription factor (T-bet, GATA3, RORgt and FOXP3, respectively (10)) and by the production of a unique set of cytokines.

TH1 cells characteristically produce interleukin-2 (IL-2) and interferon-γ (IFN-γ); they induce macrophage activation and are very effective in controlling infection by intracellular pathogens. In contrast, T_{H2} cells secrete interleukin-4, interleukin-5, and interleukin-13, and are excellent helpers for B cells in producing antibodies. T_{H17} cells, which owe their name to their production of interleukin-17, are critical for host defence against bacterial, fungal and viral infections (11).

Another highly specialized subpopulation of T_H cells is that of regulatory T cells (T_{reg}), which are responsible for the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes. It is now recognized that T_{reg} have a crucial role in the maintenance of peripheral self-tolerance, transplantation, allergy and tumour and microbial immunity (12).

Recently, a fifth subset of effectors T_H cells, called follicular helper T cell (T_{FH}) cells has been discovered and studied. This cell type is characterized by their location in the germinal centres of secondary lymphoid organs and by their ability to interact with B cells, supporting B-cell survival and antibody responses (13).

In recent years, an increasing attention has been paid to the role of T_{FH} and T_{reg} in regulating the immune response in normal and pathological conditions, suggesting that T_{reg} and T_{FH} may be excellent targets for the investigation, diagnoses, treatment and prevention of immunological disorders.

3. Regulatory T cells (T_{reg})

3.1 T_{reg} and FOXP3

Immunological tolerance requires a controlled balance between maintaining peripheral tolerance to auto antigens and preserving the potential to initiate protective immune responses against infectious agents. To achieve a fine balance between these two different immunological outcomes, a network of T_{reg} is necessary.

T_{reg} are cells specialized in suppressing excessive or misguided immune responses that can be harmful to the host, for example in autoimmune diseases, allergy or in certain

inflammatory diseases (14). On the other hand, an increased T_{reg} response can hamper host protective immunity in infectious diseases and cancer (15).

In normal individuals, T_{reg} represent about 5-10% of total CD4+ T cells in thymus, peripheral blood, and lymphoid tissues and are distinguished by their expression of interleukin 2 receptor α -chain (CD25) and the transcription factor forkhead box protein P3 (FOXP3).

The importance of this cell type was made evident by the fact that the depletion of this population in normal rodents produces a variety of autoimmune inflammatory diseases, whereas reconstitution with CD4+ CD25+ T cells can inhibit disease development (16).

The identification of FOXP3 as the master regulator gene for T_{reg} has helped in the understanding the function and this cell type. Humans with a mutation in the *FOXP3* gene develop IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). This is a severe multiorgan autoimmune disease characterized by type-1 diabetes, inflammatory bowel disease and severe allergy (17, 18). Similarly, scurfy mice, which lack FOXP3 expression, develop a lethal autoimmune syndrome (19). Further evidence for FOXP3 as a key controller of the development and suppressive function of T_{reg}, come from experiments in which transduction of the gene is sufficient to convert naïve T cell into T_{reg}-like cells (20).

FOXP3 is able to control the expression of several characteristic genes coding for cell surface molecules, such as CD25, the glucocorticoid-induced TNF receptor (*GITR*) and the cytotoxic associated antigen-4 (*CTLA-4*), and together with other proteins, is responsible for the repression of *IL-2* and other cytokine genes (21). The indispensable role of *FOXP3* for the control of these autoimmune and inflammatory disorders underlines the crucial importance of FOXP3+ T_{reg} for self-tolerance and immune homeostasis.

3.2 Markers of T_{reg}

T_{reg} are classically defined by their constitutive expression of CD25. Among the other major surface markers, CTLA-4, GITR, CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5´-nucleotidase) are the most important

markers with respect to development and function of T_{reg} (22). However, none of these markers are really specific, as they can also be expressed in activated T cells. At the moment, the expression of the transcription factor FOXP3 is the most definitive signature of T_{reg}.

3.3 T_{reg} development

T_{reg}, like all T cells, arise from progenitor cells in the bone marrow and either develop in the thymus (naturally occurring T_{reg}, nT_{reg}) or can be induced by other T cells in the periphery (inducible/adaptive T_{reg}, iT_{reg}) (Figure 1). nT_{reg} develop directly from CD4+ T-cell precursors by a process of positive selection through their interaction with thymus epithelium and dentritic cells (DCs) in the presence of major histocompatibility complex class II (MHC II) and self-peptide. These nT_{reg}, constituting 5-10% of total lymphocytes, then enter peripheral circulation and are widely distributed in peripheral lymph nodes and spleen (23) where they act to control self-reactive T cells that have escaped thymic negative selection. nT_{reg} have a polyclonal T cell receptor (TCR) repertoire that allow them to recognize a wide spectrum of self and non-self antigens (24).

In recent years it has become evident that FOXP3+ T_{reg} could also be generated outside the thymus under a variety of conditions. The iT_{reg} development *in vivo* is still not well understood, but it is known that iT_{reg} could be derived from naive T cells under the influence of various inductive signals such as TCR or cytokines such as the transforming growth factor beta (TGF-β) and IL-2 (Figure1). Under these conditions, iT_{reg} mature in peripheral sites, including mucosa-associated lymphoid tissue (MALT), where they acquire the expression of markers typical of T_{reg}, including CD25, CTLA4 and GITR. Upon upregulation of the transcription factor FOXP3, T_{reg} start to exert their suppressive effect. This includes the secretion of cytokines including interleukin-10 (IL-10) and TGF-β, which may induce cell-cycle arrest or apoptosis in effector T cells (T_{H1},T_{H2} and T_{H17}), and block co-stimulation and maturation of DCs (25). iT_{reg} are essential in mucosal immune tolerance and in the control of severe chronic allergic inflammation, and most likely they are one of the main barriers to the eradication of tumours (26).

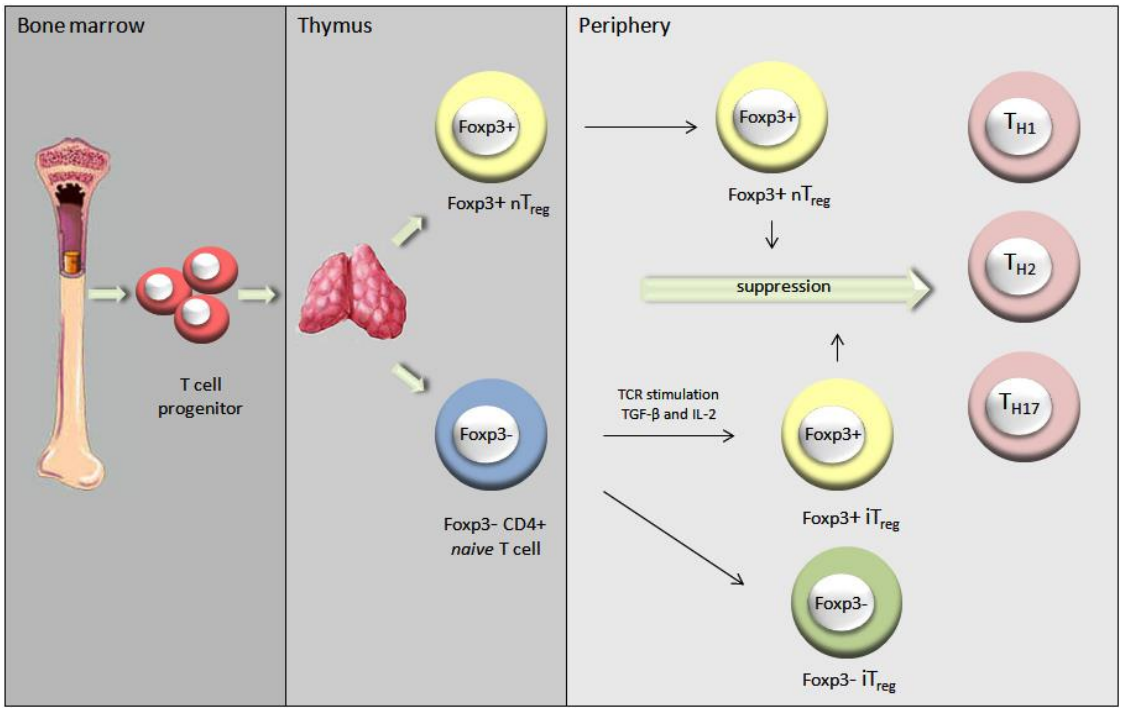


Figure 1. T_{reg} development
Hematopoietic progenitors are generated in the bone marrow and then travel to the thymus. In the thymus, high affinity TCR-MHC interactions promote the generation and expansion of natural regulatory T cells (nT_{reg}) that are released into circulation. In the periphery iT_{reg} cells are originated by FOXP3- CD4+ naïve T cells in the presence of cytokines such as TGF-β and IL2. T_{reg} then suppress the activation and proliferation of many cell types including T_{H1}, T_{H2}, and T_{H17} cells.

3.4 T_{reg} function

FOXP3+ T_{reg} can both directly and indirectly suppress the activation and proliferation of many cell types, including other T cells, B cells, DCs and NK cells. Although many possible mechanisms for T_{reg}-mediated suppression have been proposed in the past, the suppressive activity of T_{reg} remains poorly understood.

Several *in vivo* and *in vitro* findings indicate that T_{reg} suppression may operate either in a contact-independent or dependent manner (27) (Figure 2). One mechanism of contact-independent suppression has been described in several reports indicating that cytokines such as interleukin 10 (IL-10), interleukin 35 (IL-35) and TGF-ß are needed to mediate suppression (28). IL-10 and TGF-ß cytokines hamper the antigen presenting ability by down-regulating the MHC class II and co-stimulatory molecules on DCs, thus preventing the maturation and activation of DCs cells both in humans and in mice (29) (Figure 2A).

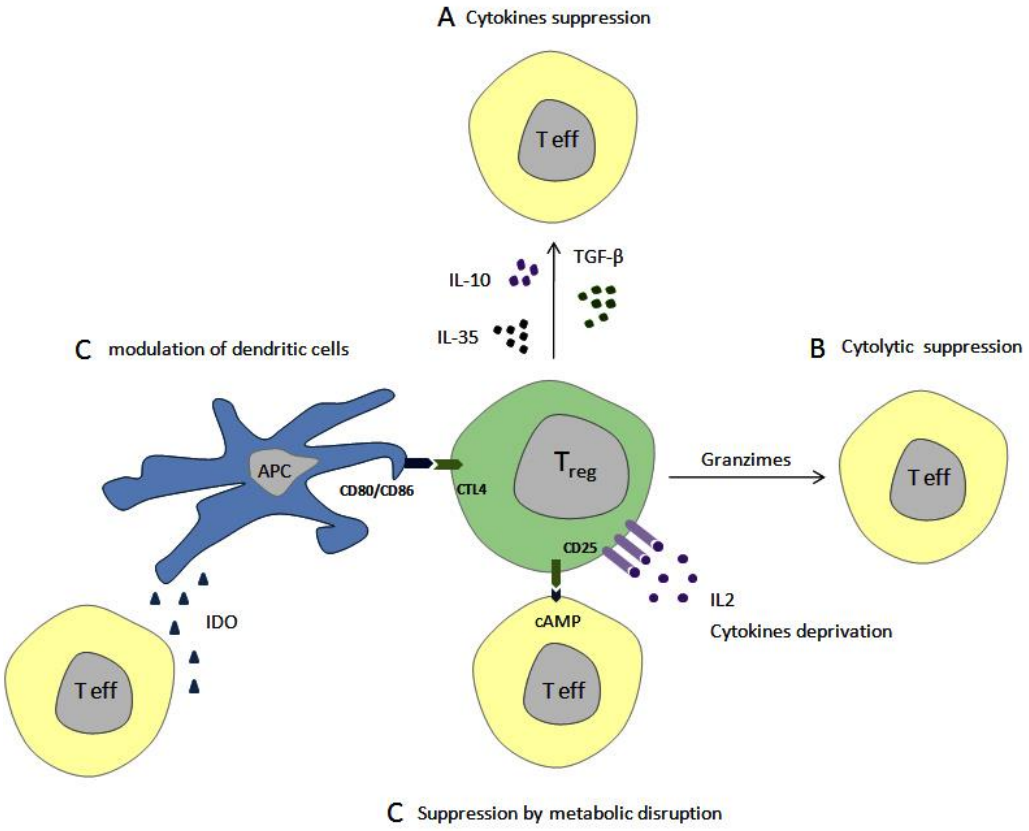


Figure 2. Basic mechanisms of T_{reg} suppression
A: T_{reg} secrete suppressor cytokines that can directly inhibit effector T cells (T_{eff}) function **B**: Activated FOXP3+ T_{reg} may function as cytotoxic cells expressing granzymes and kill T_{eff} cells. In addition, CD25 and the IL-2 receptor α chain, has the capacity to compete with T_{eff} cells for IL-2, resulting in reduced activation and expansion of T cells. **C:** T_{reg} can deliver a negative signal to T_{eff} cells through up-regulation of intracellular cyclic AMP, which leads to inhibition of T cells. **D:** CD80/CD68 molecules expressed by antigen presenting cells (APC) interact with CTLA-4 on T_{reg} resulting in IDO production leading to immunosuppressive effects.

Cytosolic activity has also been proposed as a possible mechanisms used by T_{reg} to suppress immune response. After activation, T_{reg} may express granzymes and kill activated CD4+ and CD8+ T cells by a perforin dependent mechanism (30) (Figure 2B). In addition, there is a possibility that the lack of production of IL-2 by FOXP3+ T_{reg}, together with a high expression of IL-2 receptor (IL-2R), makes T_{reg} able to exhaust IL-2 from the surrounding microenvironment, thereby contributing to reduce the activation and expansion of effector T cells (T_{eff}) (31).

In addition to contact-independent suppression, other mechanisms of contact-dependent suppression by T_{reg} have been proposed. Thus, T_{reg} can deliver a negative signal to effector T cells (T_{eff}) through up-regulation of intracellular cyclic AMP, which leads to inhibition of T cell proliferation and IL-2 formation (Figure 2C)(32). T_{reg} can also

interact with APC cells through CTLA4/CD80/CD86 interaction, resulting in the induction of the enzyme indoleamine 2,3-dioxygenase (IDO), which is a potent immunosuppressive molecule (33) (Figure 2D).

Taken together, these findings suggest that multiple mechanisms may operate in T_{reg} mediated suppression and that various molecules may be secreted or expressed on the cell surface of T_{reg} and directly contribute to their suppressive functions.

Understanding the mechanisms by which T_{reg} exert their influence is an area of intense research with broad implications for the development of therapeutic strategies for many disease processes including cancer, diabetes, and immune mediated diseases.

3.5 T_{reg} and cancer

Understanding the mechanisms of tumour tolerance is a major challenge for cancer research. It is becoming increasingly clear that T_{reg} play an active and significant role in the progression of cancer, and have an important role in suppressing tumour-specific immunity (23, 34).

Over the last years a number of reports have described the presence of tumour-specific immune cells in various mouse and human malignancies (35). In the presence of Treg, the immune system frequently fails to prevent tumour formation and metastasis, and it can contribute to tumour growth and progression. In breast (36), ovarian (37) and gastric cancer (38), the presence of high number of infiltrating T_{reg} have been associated with poor survival and tumour relapse. Also, targeting T_{reg} by administration of a CD25 monoclonal antibody abrogated immunologic unresponsiveness to tumours and induced spontaneous development of tumour-specific CD8+ T_{eff} cells and NK cells (23).

Regarding how T_{reg} population increases in tumours, several possibilities have been postulated. One of the possible mechanisms for T_{reg} cells recruitment to the tumour site may involve the production of the chemokine (C-C motif) ligand 22 (CCL22) by tumour cells and tumour infiltrating macrophages, which chemoattract and recruit CD4+ CD25+ T_{reg} expressing C-C chemokine receptor type 4 (CCR4) (Figure 3). On the other hand, an increased T_{reg} recruitment could be promoted by proliferating and dying tumour cells that provide a large amount of self-antigens (39).

It is also likely that FOXP3+ T_{reg} may be derived from naïve CD4 T cells due to high concentrations of TGF-β secreted by tumour cells or DCs present in tumours (40).

Finally, T_{reg} can inhibit the generation of T_{eff} cells, resulting in the induction of tolerance against the tumour.

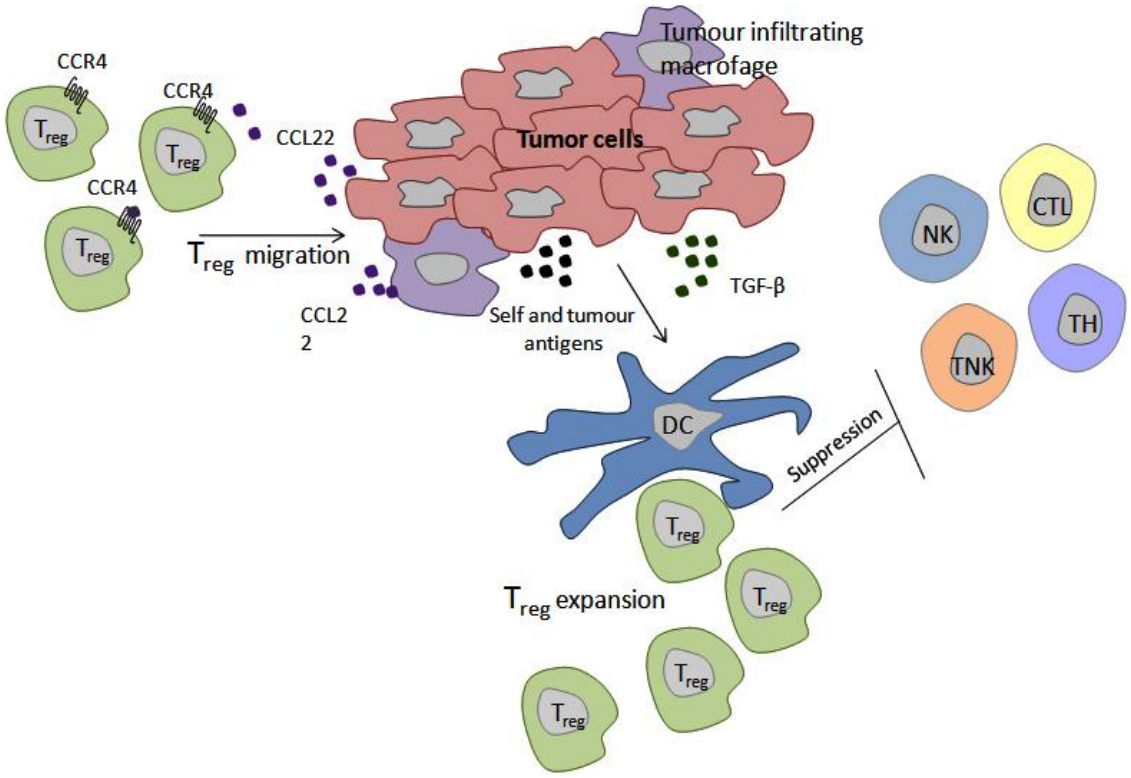


Figure 3. T_{reg} suppress antitumor immune responses
Both tumour cells and tumour-associated macrophages produce the chemokine CCL22 that attract T_{reg} through the CCR4 receptor, leading to the accumulation of T_{reg} in the tumour microenvironment (T_{reg} migration). T_{reg} receive proliferative signals (iT_{reg} expansion) through the presence of tumour-associated self-antigens and high levels of TGF-β cytokine. Also, dendritic cells (DCs) may further enhance T_{reg} expansion. The increased number of T_{reg} inhibits natural killer cells (NK), natural killer T cells (NTK), CD8+ cytotoxic T lymphocytes (CTL) and CD4+ helper T cells, and could contribute to tumour progression. This image was modified from reference (41).

The effect that T_{reg} play on tumour-specific T cell immunity in lymphomas is less well established. Recent evidence suggests that the cellular composition of the tumour microenvironment, particularly the quantity of tumour-infiltrating T_{reg}, can significantly modify the clinical outcome in hematologic malignancies. Contrary to what has been observed in solid tumours, in classical Hodgkin's lymphomas (cHL) (42) and in follicular lymphomas (FL) (43), the presence of a high number of T_{reg} correlate with better response and improved survival. These studies suggest that tumour-infiltrating T_{reg} in

lymphoma may act to suppress not only tumour-infiltrating cytotoxic cells, but also the malignant cells. The implication of T_{reg} in the behaviour and development of these pathologies may be therefore more complex than for solid tumours, and has to be further investigated.

4. Follicular helper T cells (T_{FH})

4.1 Follicular helper T cells as a distinct T_H subset

T_{FH} are essential moderators of the immune response. Although they do not have the capability to directly target invading pathogens, they can orchestrate a coordinated response from many other cell types.

T_{FH} are a subset CD4+ T cells localized in the B cell follicles of secondary lymphoid organs such as lymph nodes, spleens and Peyer's patches. T_{FH} are best defined by their expression of the C-C chemokine receptor type 5 (CXCR5) that allows them to migrate into the B cell follicles of secondary lymphoid tissues, where they provide help for the differentiation of B cells (9, 10). Gene expression analysis of purified T_{eff} cells expressing high levels of CXCR5 revealed a strikingly different gene expression profile from T_{H1}, T_{H2}, T_{H17} and T_{reg}, supporting the theory that T_{FH} are a distinct subset of CD4+ T cells (44, 45). Compared to other T-cell subsets, T_{FH} cells show a unique transcript signature characterized by the expression of CD4, CD10, CXCR5, B-cell lymphoma 6 (BCL6), CD40L and CD57 (which are also present in B-cells and other types of T-cells) and by molecules that appear to be more restricted to T_{FH} cells (Figure 4). The molecules hat are important for the role of T_{FH} cells helping B cells differentiation in germinal-centers include the inducible T-cell co-stimulator (ICOS), signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) and programmed cell death 1 (PD-1) (Figure 4). The transcription factor BCL6 and the

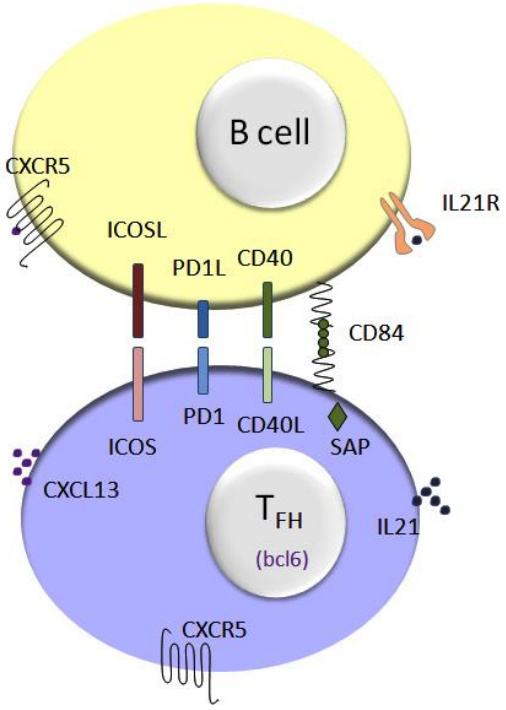


Figure 4. Interaction between T_{FH} and B cells
Interactions between T_{FH} and B cells are mediated by an array of accessory molecules, including ICOS, PD-1, CD40L and SAP on T_{FH}, and by ICOSL, PD1L, CD40 and the SLAM family of receptors (CD84) on B cells. T_{FH} are also characterized by the expression of BCL6 protein and the production of IL-21.

cytokine IL-21 have also important roles in T_{FH} cell differentiation and function, and both of them are now considered hallmarks of this cell type.

4.2 T_{FH} development

The mechanism of T_{FH} cell generation, including the signals required for T_{FH} cell differentiation and the time at which T_{FH} cell differentiation occurs, are still not well understood. One of the possible scenarios is illustrated in Figure 5.

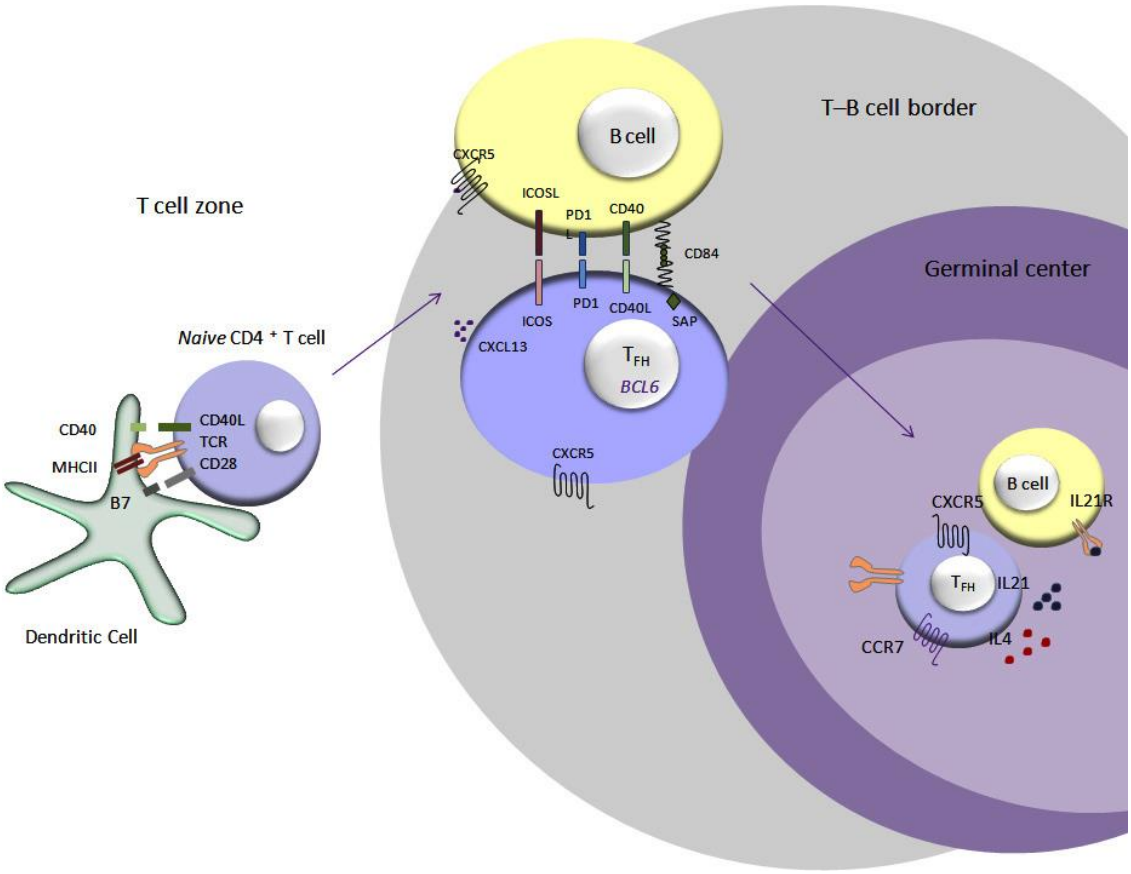


Figure 5. Multiple steps for the generation of T_{FH}
In the T cell zone of secondary lymphoid organs, DCs cells activate naive $CD4^+$ T cells. T cell receptor signals, together with co-stimulation provided by CD28/B7, CD40L/CD40, ICOS/ICOSL and PD-1/PD-1L interactions are able to initiate the program of T_{FH} differentiation. Increased expression of CXCR5 helps T_{FH} to localize to B-T cell border where they interact with B cells trough CD40L/CD40, ICOS/ICOSL, CD84/SAP and PD-1/PD-1L. T_{FH} then migrate in the GC where they provide help for the differentiation of B cells.

Upon antigen encounter, DCs migrate from the periphery to the T zone of the lymphoid tissue, where they present the antigen, by means of peptide MHC class II complex.

Naïve CD4+ T cells, with a T cell receptor (TCR) specific for the presented antigen, will form a stable contact with the DCs and receive signals through co-stimulation provided by CD28/B7, CD40L/CD40, ICOS/ICOSL and PD-1/PD1L interaction, and by the up-regulation of the transcriptional repressor BCL6 (46). As they do this, activated T cells down regulate the chemokine C-C motif receptor 7 (CCR7) and up-regulate CXCR5, and then move towards the follicle to the T-B border where they encounter B cells and will receive a second round of activation signals (25).

The importance of B cells in T_{FH} development is demonstrated by the lack of T_{FH} in the absence of B cells, or when their interaction is abolished (47). Stable contacts between T and B cells in the T-B cell border are initially mediated through TCR and peptide MHC complex. Once again, a range of cell surface receptors interaction including CD40/CD40L, ICOS/ICOSL, SLAM family members (SLAMF) on both cell types, and PD-1/ PDL1 are crucial to complete and maintain T_{FH} differentiation (48).

These interactions culminate in the secretion of cytokines by T_{FH}, particularly IL-4 and IL-21, and the migration of T_{FH} into the germinal centre (GC) (49). Activated T cells that fail to get the necessary signal do not enter the follicles.

4.3 SAP and PD-1 in T_{FH} cell development

It has also become clear that the SLAM family of surface receptors play an important role in T_{FH} generation. The importance of these molecules in T-B interactions first came to light in patients suffering from the immunodeficiency X-linked lymphoproliferative disease (XLP). XLP is caused by mutations in the gene encoding SAP *(SH2D1A*), a cytoplasmic adaptor molecule that signals downstream of the SLAM family of receptors. Patients with XLP, as well as gene-targeted KO mice that lack SAP expression, display a deficiency in T-dependent B cell responses (50, 51).

Several recent studies have demonstrated that SAP, acting downstream of CD84, is required to regulate adhesion between T and B cells. Therefore, in the absence of SAP or CD84, CD4+ T cells are unable to form stable conjugates with B cells, and this leads

to a decrease in signals from T_{FH} co-stimulatory molecules and cytokines, which directly impact B cell differentiation and survival (52).

Another molecule highly expressed by T_{FH} is PD-1 (CD279). PD-1 molecule and its ligands, programmed death ligand 1 and 2 (PD-L1 and PD-L2) have an important inhibitory function in the regulation of immune homeostasis and in the maintenance of peripheral tolerance (53). Evidence that PD-1 suppresses activation of the immune response comes from studies in which PD-1-deficient mice develop spontaneous autoimmune diseases such as systemic lupus erythematosus, some dilated cardiomyopathy, rheumatoid arthritis and type1 diabetes mellitus, due to the uncontrolled persistent T cell activation (54).

Although PD-1 is highly expressed in T_{FH}, little is known about its role in the development or function of this cell type. PD-1 is expected to provide an inhibitory signal to T_{FH}, preventing excess of CD4+ T cells proliferation in the GC (55, 56). Additionally, it is thought that the induction of PD-1 by T_{FH} cells within the GC promotes survival and selection of B-cells with high affinity immunoglobulin receptor (57).

4.4 T_{FH} function

T_{FH} are distinguished from other helper subsets by their unique ability to localise into B cell follicles and by their capacity to provide help to B cells to support their activation, expansion, differentiation and GC formation (58).

The best-characterized B cell helper signal provided by T_{FH} cells is through CD40L, which is highly expressed in these cells. CD40L is a potent activator of B cells and is able to induce proliferation and, in combination with cytokines, isotype switching and B cell differentiation (59). The importance of this molecule for B cell responses has been demonstrated in mice lacking CD40 or CD40L, which display abortive B cell responses and a failure to generate GCs and long-term memory B cells (60).

Another mechanism by which T_{FH} regulates B cell responses is through the secretion of cytokines. T_{FH} are characterized by expression of IL-21, a cytokine capable of modulating B cell differentiation and proliferation (61). Recently, other cytokines (IL-4, IL-10, IL-17 and IFN-γ) have been described to be secreted by T_{FH} and to play a role in isotype switching and antibody production (62)

4.5 Implications of T_{FH} cells in lymphomas

It is now well established the important role that T_{FH} play in the GC microenvironment and in B cell development, and there is also emerging evidence that the aberrant accumulation or deregulation of T_{FH} can also lead to autoimmune disorders (63).

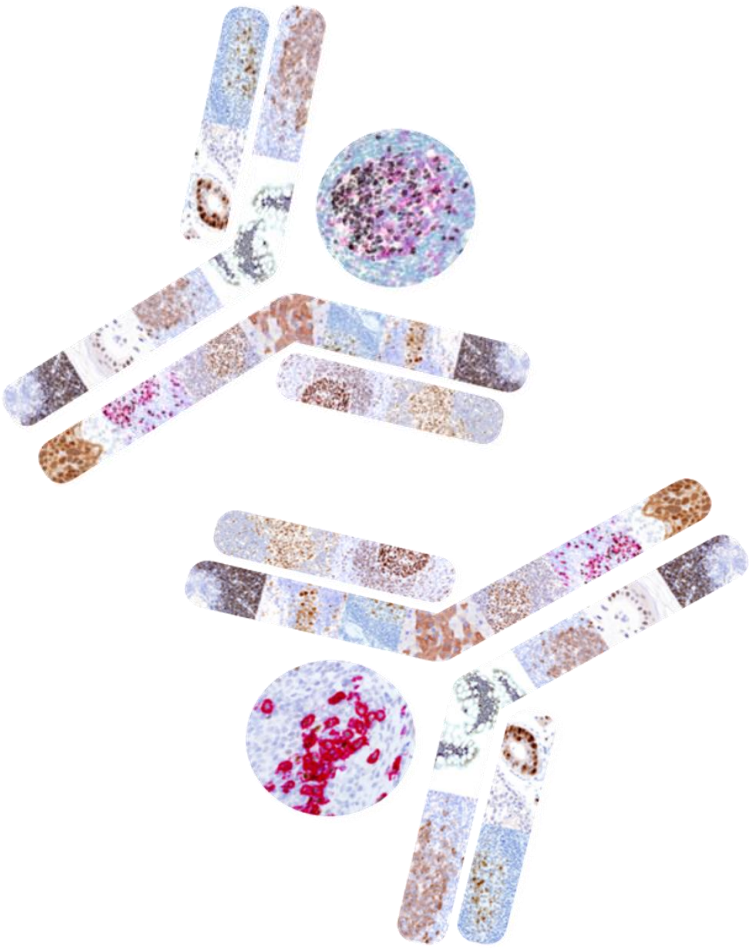
Recent studies are also suggesting that T_{FH} cells may be important players in the biology of the tumour development and in particular in lymphoproliferative disorders (64).

The GC microenvironment is not only an essential niche for generation of B-cell response, but it is also considered to be critical in the development of most human lymphoid neoplasms. Although most lymphomas originating from GC lymphocytes are B-cell lymphomas, it has been suggested that some peripheral T-cell lymphomas, in particular AITL, may arise from GC T_{FH} cells. These results have been confirmed by GEP expression profiling studies carried out on peripheral T-cell lymphomas, which have identified that T_{FH} represent the putative cell of origin of AILT, being both PD-1 and CXCL13 identified as markers for this T cells subset (44).

Recent studies have highlighted that T_{FH} markers are not exclusive of AITL, since they can also identify tumour cells in other type of T cell lymphomas such as cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma (CSTCL). This finding suggests that similar process of lymph node B-cell stimulation by F_{TH} could also take place outside the GC (65).

In recent years, several studies have highlighted the presence of T_{FH} cells in the microenvironment of a variety of B cell lymphomas, stressing the importance of the identification of T_{FH} as an additional tool for lymphoma diagnosis (64). In addition, the presence of specific T_{FH} in the tumour microenvironment could directly influence the tumour development and could help in predicting the response to treatment and survival (64). An example is the presence of T_{FH} cells in nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL), a slow-growing type of lymphoma characterized by the presence of a small population of neoplastic B cells within a lymphocyte and histiocyte rich background. The distribution of T_{FH} cell in this type of tumours is not random, since PD-1positive T_{FH} cells form rosettes around the neoplastic B cells, where they are likely supporting tumour growth and survival (66).

Follicular lymphoma (FL) is a neoplasm derived from follicular GC cells, where tumour cells proliferate in follicular structures in close association with T_{FH} and follicular DCs (67). A recent study has demonstrated that the number of PD1 positive cells infiltrating the tumour is a predictor of survival in patients with FL, suggesting that PD-1 is able to identify an important subset of T_{FH} in the microenvironment of FL that could directly participate in the modulation of the tumour cell behaviour and consequently influence clinical evolution (68)



INTRODUCCIÓN

1. Linfomas T: Búsqueda de nuevos biomarcadores

Los linfomas son un grupo heterogéneo de tumores que se originan como resultado de la transformación neoplásica de los linfocitos B y T durante diferentes estadios de su diferenciación celular. La gran variedad de neoplasias linfoides existente constituye un reflejo de las diferentes etapas de desarrollo linfocitario y de la complejidad del sistema inmune. Las características clínicas y patológicas de las neoplasias linfoides se encuentran recogidas en la clasificación de la Organización Mundial de la Salud (OMS) de los tumores de tejidos hematopoyéticos y linfoides, que utiliza la información disponible (morfológica, citoquímica, inmunofenotípica, genética y clínica) para definir las distintas entidades linfoides con significado clínico (1). La clasificación de la OMS divide los linfomas en tres grupos: linfomas de células B, linfomas de células T y "*natural killer*" (NK) y linfomas de Hodgkin.

A diferencia de los importantes avances alcanzados en el conocimiento y clasificación de los linfomas de células B, el progreso realizado en el campo de los linfomas de células T ha sido mucho más lento, por lo que hacer frente a este tipo de linfomas sigue siendo un reto, tanto desde el punto de vista diagnóstico, como histopatológico y terapéutico.

Las neoplasias malignas derivadas de las células T maduras y NK (también llamados linfomas T periféricos (PTCL)), constituyen un grupo heterogéneo de neoplasias poco frecuentes, representando alrededor del 15% de todos los linfomas no Hodgkin en todo el mundo. Además, con pocas excepciones, los PTCL son resistentes a los protocolos clásicos de quimioterapia y muestran una peor evolución clínica (2).

El diagnóstico histopatológico de los linfomas T es a menudo difícil, debido a la amplia variedad morfológica, inmunofenotípica y a los solapamientos existentes entre las diferentes entidades de células T (2). De forma reciente, mediante perfiles de expresión génica se han obtenido nuevos conocimientos sobre la biopatología de los linfomas T, que han conducido a la identificación de nuevos biomarcadores con relevantes implicaciones diagnósticas, pronósticas y terapéuticas (3, 4). Basándose en este tipo de estudios, se han identificado marcadores específicos del subtipo más común de PTCL, el linfoma T angioinmunoblástico (AITL). De forma más concreta, se ha identificado como célula de origen del mismo un subtipo de células T CD4+

denominada célula T colaboradora folicular (T_{FH}). Estos linfocitos se localizan en el centro germinal (GC) realizando funciones auxiliares sobre los linfocitos B foliculares (5).

Así mismo, varios estudios han puesto de relieve la importancia de otro subtipo de células T CD4+ en los procesos linfoproliferativos, las células T reguladoras (T_{reg}). Este subtipo celular se ha convertido en un foco de interés debido a la función que ejercen en la modulación de la respuesta inmune, concretamente en la supresión de los linfocitos antígeno-reactivos asociados al tumor (6). La identificación y producción de marcadores específicos capaces de identificar los subtipos celulares T podría ser muy útil para ayudar a discernir el papel que juegan las células T_{reg} y T_{FH} en el desarrollo tumoral.

La calidad diagnóstica y la posibilidad de clasificación de los tumores linfoides mejoraron sustancialmente en la década de los 80 gracias a la labor de numerosos investigadores, destacando entre ellos el profesor David Mason (7), gracias a cuyos trabajos se desarrollaron métodos inmunohistoquímicos empleando una amplia variedad de anticuerpos monoclonales contra antígenos linfoides de la superficie celular. Esta nueva metodología se adaptó a la rutina de laboratorio empleando muestras fijadas en formol e incluidas en parafina (FFPE), lo que implicó un gran avance en la histopatología (8).

Esta tecnología fue rápidamente adoptada por los patólogos, tanto en centros académicos como en hospitales y se utiliza en la actualidad, junto a la morfología y a los estudios moleculares, para la clasificación y diagnóstico de las neoplasias linfoides.

El objetivo de esta tesis es la identificación de nuevos marcadores de diferenciación específicos de linfocitos T y en concreto de las células T_{reg} y T_{FH}. Los anticuerpos obtenidos frente a estos marcadores serán fundamentales para estudiar el comportamiento biológico del tumor y permitirán la detección de estos subtipos celulares en muestras de tejido FFPE. Así mismo, estos nuevos marcadores podrán ayudar tanto en el diagnóstico como en el pronóstico de los pacientes con linfoma T.

2. Linfocitos T colaboradores (T_H)

Las células T CD4+ constituyen uno de los principales componentes de la respuesta inmune, controlando los procesos asociados a dicha respuesta contra las infecciones. Estas células no tienen actividad citotóxica ni son responsables de eliminar las células infectadas o los patógenos de forma directa, sino que estimulan a otras células para realizar dichas funciones.

Los linfocitos T "*naive"* o vírgenes acceden a los órganos linfoides secundarios, en las áreas donde se localizan las células T, y tras la interacción con las células presentadoras de antígeno (APC), se diferencian en distintas subpoblaciones de linfocitos T colaboradores (efectores) CD4+ (T_H), denominados T_{H1}, T_{H2}, T_{H17} y T_{reg} (9). Cada subpoblación está genéticamente regulada por un único factor de transcripción "*master*" o patrón (T-bet, GATA3, RORgt y FOXP3 respectivamente (10)) y por la producción de un conjunto de citoquinas específicas.

Las células T_{H1} producen interleuquina-2 (IL-2) e interferón-γ (IFN-γ), las cuales inducen la activación de macrófagos y son muy eficaces en el control de las infecciones producidas por patógenos intracelulares. Por otro lado, las células T_{H2} secretan IL-4, IL-5 e IL-13 y son excelentes colaboradoras de las células B en la producción de anticuerpos. Las células T_{H17}, denominadas así dada su producción de IL-17, son fundamentales para la defensa del huésped contra las infecciones bacterianas, fúngicas y virales (11).

Otra subpoblación muy especializada de células T_H, es la constituida por las células T reguladoras (T_{reg}), un subconjunto de linfocitos T responsable del mantenimiento de la auto-tolerancia inmunológica mediante la supresión de los linfocitos auto-reactivos. Actualmente se sabe que las T_{reg} tienen un papel crucial en el mantenimiento de la auto-tolerancia periférica, los trasplantes, la alergia y la inmunidad tumoral y microbiana (12).

De forma reciente ha sido descubierta una quinta subpoblación de células T colaboradora, las denominadas células T colaboradoras foliculares (T_{FH}). Este tipo de células se caracteriza por su localización en los GC de los órganos linfoides secundarios y su capacidad de interactuar con las células B, interviniendo tanto en la supervivencia de las mismas como en la respuesta mediada por anticuerpos (13).

En los últimos años se ha prestado una mayor atención al papel de las células T_{FH} y T_{reg} en la regulación de la respuesta inmune tanto en condiciones fisiológicas como patológicas, siendo estas células excelentes candidatas para la investigación, diagnóstico, tratamiento y prevención de determinados trastornos inmunológicos.

3. Células T reguladoras (T_{reg})

3.1 T_{reg} y FOXP3

El mantenimiento de la tolerancia inmunológica requiere la existencia de un equilibrio entre el mantenimiento de la tolerancia periférica a los antígenos propios y la respuesta inmunitaria protectora contra agentes infecciosos. Para lograr un buen equilibrio entre estos dos procesos es necesaria la existencia de los linfocitos T_{reg}.

Los linfocitos T_{reg} están especializados en la supresión de la respuesta inmune excesiva o anómala que puede llegar a ser dañina para el huésped al derivar en procesos patológicos tales como enfermedades autoinmunes, alergias y determinadas enfermedades inflamatorias (14). Por otro lado, una respuesta exagerada por parte de los mismos puede llegar a alterar la función inmunológica "habitual" del huésped frente a enfermedades infecciosas y al cáncer (15).

En los individuos sanos, las células T_{reg} representan el 5-10% del total de las células T CD4+ de timo, sangre periférica y tejidos linfoides. Estas células se caracterizan por la expresión, tanto del receptor α de la interleuquina 2 (CD25) como del factor de transcripción FOXP3 (*forkhead box protein* P3).

La importancia de este tipo de células se hizo patente al observar que la disminución de esta población en roedores producía una gran variedad de enfermedades inflamatorias autoinmunes, mientras que la reconstitución con células T CD4+ CD25+ inhibía el desarrollo de dichas enfermedades (16).

La identificación de *FOXP3* como el gen regulador "*master*" de las células T_{reg} ha contribuido a la comprensión y al estudio de la función de este tipo de linfocitos.

En humanos, la mutación del gen *FOXP3* se ha asociado al desarrollo de un síndrome denominado IPEX (Inmuno-desregulación, Poliendocrinopatía y Enteropatía ligada al cromosoma X), una severa enfermedad multiorgánica y autoinmune caracterizada por diabetes tipo 1, enfermedad inflamatoria intestinal y alergia severa (17, 18). De forma

similar, los ratones "*scurfy*", que carecen de la expresión de la proteína FOXP3, desarrollan un síndrome autoinmune letal (19). El papel clave de FOXP3 en el desarrollo y función supresora de las T_{reg} ha sido ulteriormente confirmado en experimentos en los que la traducción del gen *FOXP3* es suficiente para convertir células T "*naive*" o vírgenes en células con funciones similares a la T_{reg} (20).

Además, FOXP3 es capaz de controlar la expresión de varios genes de moléculas de superficie, tales como CD25, la familia de genes reguladores GITR (*Glucocorticoid-Induced TNF Receptor*) y CTLA-4 (*CTL-associated Antigen-4*), y junto con otras proteínas es responsable de la represión de los genes de la IL-2 y otras citoquinas (21).

Todos los estudios realizados hasta la fecha demuestran el papel fundamental de FOXP3 en el control de las enfermedades autoinmunes e inflamatorias y subrayan la importancia crucial de las células T_{reg} CD4+ FOXP3+ en la auto-tolerancia y la prevención de enfermedades autoinmunes.

3.2 Marcadores de las células T_{reg}

Las células T_{reg} se caracterizan por la expresión constitutiva de CD25. Además, existen otros marcadores de superficie como GITR, CTLA-4, CD39 y CD73 y niveles altos del receptor de folato 4 (FR4), cuya presencia está relacionada con el desarrollo y función de las células T_{reg} (22). Sin embargo, ninguno de estos marcadores es específico, dado que también se expresan en células T activadas, por lo que en estos momentos, la expresión del factor de transcripción *FOXP3* constituye el marcador más específico para su identificación y estudio.

3.3 Desarrollo de las células T_{reg}

Las T_{reg}, al igual que todos los linfocitos T, derivan de las células progenitoras de la médula ósea y pueden desarrollarse tanto en el timo (células T reguladoras naturales; nT_{reg}) como ser inducidas por otras células T de la periferia (células T reguladoras inducibles/adaptativas, iT_{reg}) (Figura 1). Las nT_{reg} provienen directamente de las células T precursoras CD4+ mediante un proceso de selección positiva a través de su interacción con el epitelio tímico y con las células dendríticas (DC) en presencia del complejo mayor de histocompatibilidad de clase II (MHC II) y del antígeno propio. Las

células T_{reg}, que constituyen de un 5-10% de los linfocitos CD4+ totales, pasan a continuación a la circulación periférica y se distribuyen tanto por los ganglios linfáticos como por el bazo (23), donde actúan sobre las células T autoreactivas que han escapado a la selección negativa del ti mo.

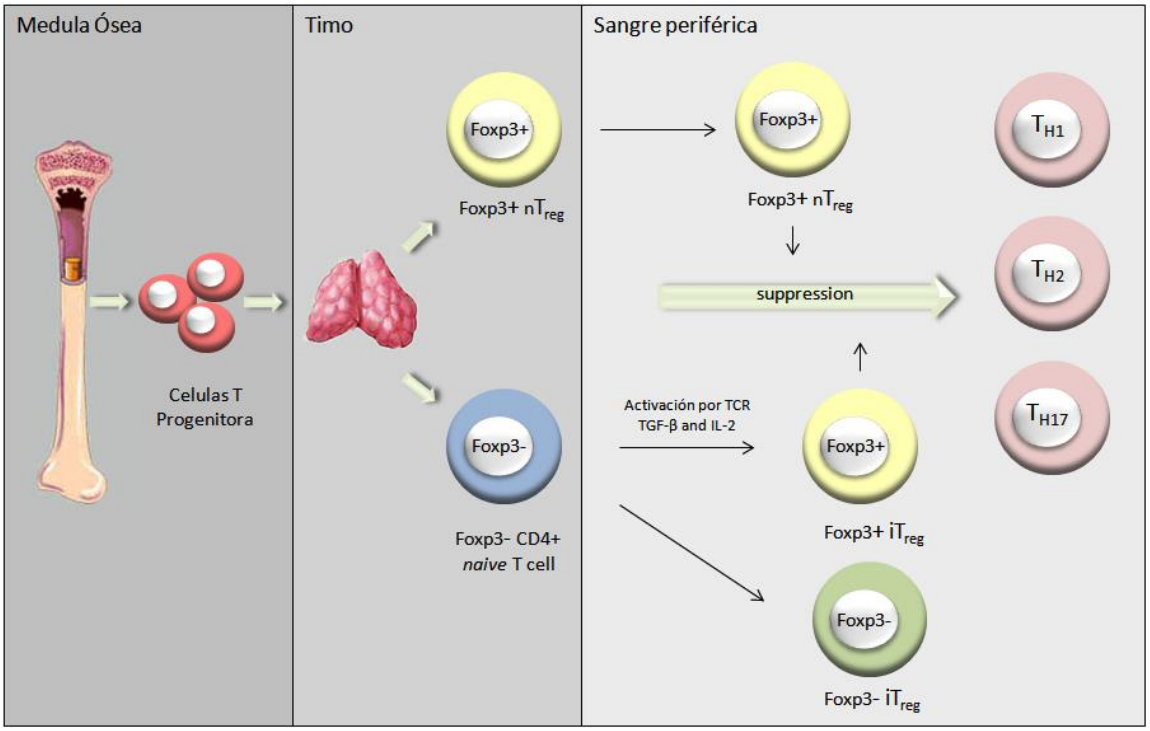


Figura 1. Desarrollo de las células T_{reg}
Los progenitores hematopoyéticos se generan en la médula ósea y posteriormente viajan al timo. En éste, las interacciones de alta afinidad tipo TCR-MHC II promueven la generación y expansión de las nT_{reg} que serán liberadas a la circulación sanguínea. En la periferia, las iT_{reg} se generan a partir de las células T CD4+ FOXP3- "*naive*" o vírgenes en presencia de citoquinas (TGF-β e IL-2). Las células T_{reg} suprimen la activación y proliferación de muchos tipos celulares, incluyendo las células T_{H1}, T_{H2} y T_{H17}.

Las nT_{reg} presentan un repertorio policlonal de receptores de células T (TCR), que les permite reconocer un amplio espectro de antígenos propios y no propios (24).

En los últimos años se ha hecho evidente que las células T_{reg} también podrían generarse directamente en la periferia bajo diferentes condiciones. El desarrollo "*in vivo*" de las células iT_{reg} todavía no ha sido completamente esclarecido, sin embargo, se postula que estas células podrían derivar de linfocitos T "*naive*" o vírgenes como consecuencia de diferentes señales inductivas, como receptores de células T o citoquinas, o del factor de crecimiento transformante beta (TGF-β) o la IL-2 (Figura 1).

Bajo estas condiciones, las células iT_{reg} madurarían en zonas periféricas, como por ejemplo en el tejido linfoide asociado a mucosas (MALT), donde adquirirían la expresión de los marcadores característicos de las células T_{reg} anteriormente citados (CD25, CTLA4 y GITR). Después, tras la activación del factor de transcripción FOXP3, las células T_{reg} comenzarían su efecto supresor. Esto incluiría la secreción de citoquinas (IL-10 y TGF-β) tanto para detener el ciclo celular como para inducir apoptosis en las células T efectoras (T_{H1}, T_{H2} y T_{H17}), y el bloqueo de la co-estimulación y maduración de las DCs (25). Las células iT_{reg} parecen ser esenciales en los procesos asociados a la tolerancia inmunológica en mucosas, en el control de la inflamación alérgica crónica severa y probablemente sean una de las principales defensas para la erradicación de los tumores (26).

3.4 Función de los linfocitos T_{reg}

Las T_{reg} FOXP3+ son capaces de suprimir, tanto de forma directa como indirecta, la activación y proliferación de muchos tipos celulares, siendo los más destacados los linfocitos T, B, las DCs y las células NK.

Aunque se han propuesto diferentes mecanismos para explicar esta supresión, no ha sido posible clarificar hasta el momento todos los detalles implicados en este proceso. Diferentes hallazgos obtenidos mediante ensayos *in vivo* e *in vitro* indican que la supresión mediada por las células T_{reg} podría ser realizada de varios mecanismos, independientes o dependiente de contacto (27) (Figura 2).

Estudios recientes han descrito un mecanismo de supresión independiente de contacto, que utilizaría diferentes tipos de citoquinas, tales como la IL-10, IL-35 y TGF-beta, como mediadores de dicha supresión (28). Según dichos estudios, tanto la IL-10 como TGF-ß intervendrían dificultando la capacidad presentadora de antígeno mediante una inhibición del complejo mayor de histocompatibilidad de clase II y de las moléculas co-estimuladoras de las DCs, evitando así la maduración y activación de las DCs, tanto en humanos como en ratones (29) (Figura 2A).

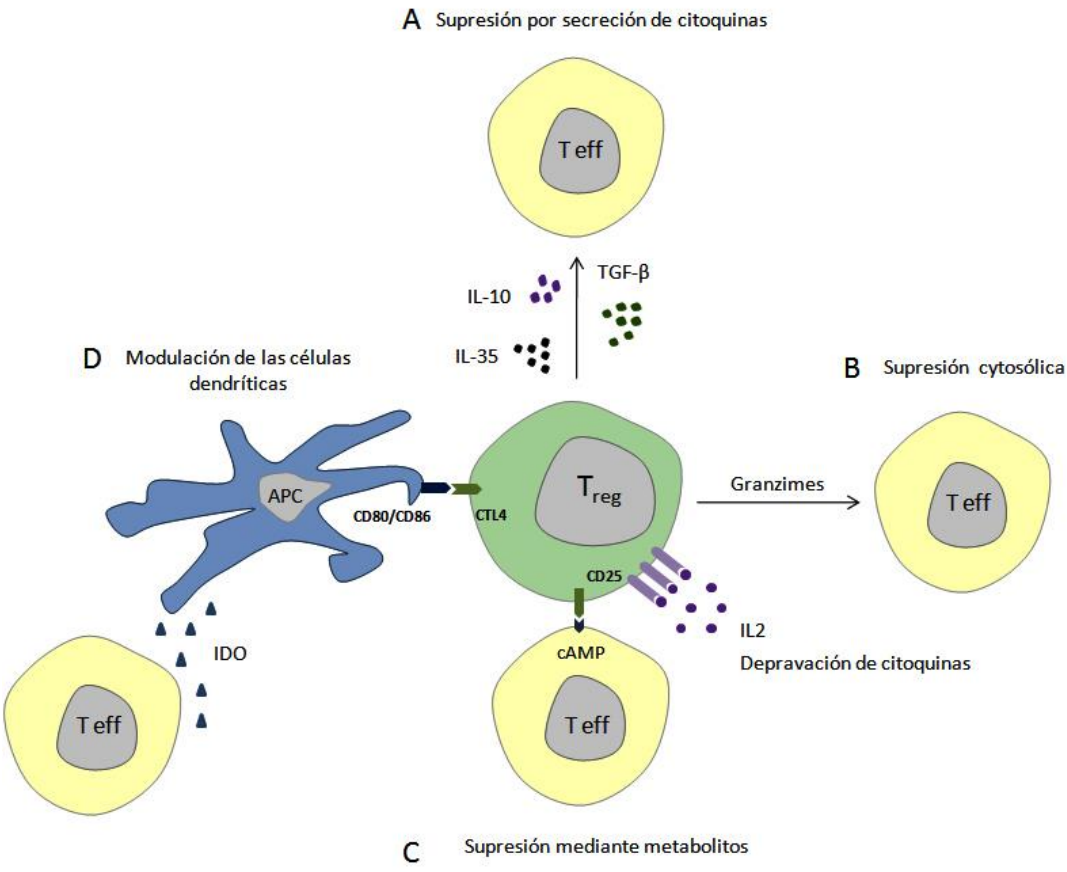


Figura 2. Mecanismos básicos de supresión mediada por las células T_{reg}
A: Las células T_{reg} secretan citoquinas supresoras que inhiben directamente las células T efectoras (T_{eff}). **B**: Las células T_{reg} FOXP3+ activadas pueden funcionar como células citotóxicas que expresan granzimas cuya función es colaborar en la eliminación de las células T_{eff}. **C:** De igual forma, la molécula CD25 (receptor alpha de la IL-2) tiene la capacidad de competir con células T_{eff} por la IL-2, lo que dificulta la activación y expansión de las células T. Las células T_{reg} también pueden inducir una señal inhibición a las células T_{eff} a través de la regulación del AMP cíclico intracelular. **D**: Las moléculas CD80/CD68 presentes en la superficie de las APC interactúan con la molécula CTLA-4 de las células T_{reg}, lo que deriva en la producción de IDO encargado de dirigir los efectos inmunodepresores.

La actividad citosólica también ha sido propuesta como uno de los posibles mecanismos empleados por las T_{reg} para suprimir la respuesta inmune. Después de su activación, las células T_{reg} expresan granzimas responsables de la eliminación de los linfocitos CD4+ activados y linfocitos T CD8+ mediante un mecanismo dependiente de la perforina (30) (Figura 2B).

Asimismo existe la posibilidad de que la falta de producción de IL-2 por parte de las células T_{reg} FOXP3+, junto con una elevada expresión del receptor de IL-2 (IL-2R), haga

que las T_{reg} consuman la IL-2 presente en el entorno, lo cual puede contribuir a reducir la activación y expansión de las células T_{eff} (31).

Además de la supresión mediada por las T_{reg} independiente de contacto, ha sido descrito otro mecanismo de supresión dependiente de contacto. En este caso, las células T_{reg} envían una señal de tipo negativo a las células T_{eff} a través del AMP cíclico intracelular, lo que conduce a la inhibición de la proliferación de las células T y a la producción de IL-2 (Figura 2C) (32).

Las T_{reg} también pueden entrar en contacto con las APC a través de la interacción CTLA4/CD80/CD86, lo que genera la inducción de un potente inmunosupresor, la enzima indolamina 2,3-dioxigenasa (IDO) (33) (Figura 2D).

En resumen, los estudios realizados sugieren que existen múltiples mecanismos implicados en la supresión mediada por las células T_{reg} y que existe una gran variedad de moléculas secretadas y/o expresadas en la superficie celular de las mismas que contribuyen directamente a sus funciones supresoras.

El conocimiento de los mecanismos por los cuales las células T_{reg} realizan su función constituye una extensa área de investigación con importantes implicaciones en el desarrollo futuro de estrategias terapéuticas para procesos patológicos como cáncer, diabetes y las enfermedades inmunológicas.

3.5 Las células T_{reg} y el cáncer

El conocimiento de los mecanismos responsables de la tolerancia frente a los tumores es uno de los grandes desafíos de la investigación contra el cáncer. Cada vez resulta más claro que las células T_{reg} desempeñan un papel activo y relevante en la progresión del cáncer y por tanto representan un factor importante en la supresión de la inmunidad específica del tumor (23, 34).

En los últimos años, una serie de investigaciones han descrito la presencia de células inmunitarias específicas del tumor, tanto en neoplasias humanas como murinas (35). En estos casos, la presencia de células T_{reg} en el microambiente tumoral podría inducir al “fallo” en el sistema inmunológico, lo que contribuiría al crecimiento y progresión del mismo. La presencia de un gran número de células T_{reg} infiltrantes se ha asociado con una peor supervivencia y recidiva tumoral en cáncer de mama (36), ovario (37) y

gástrico (38). Además, se ha comprobado que la eliminación de las células T_{reg} mediante la administración del anticuerpo monoclonal CD25 produce una mejora de la respuesta inmunológica frente los tumores y el desarrollo espontáneo de células T_{eff} CD8+ y celulas NK (23).

Para esclarecer el aumento del número de células T_{reg} en los tumores se han propuesto varias hipótesis. Una posible explicación para explicar el reclutamiento de estas células sería la producción de la citoquina CCL22 por parte de las células tumorales y macrófagos infiltrantes, que atraerían a las células T_{reg} CD25+ CD4+ CCR4+ (Figura 3).

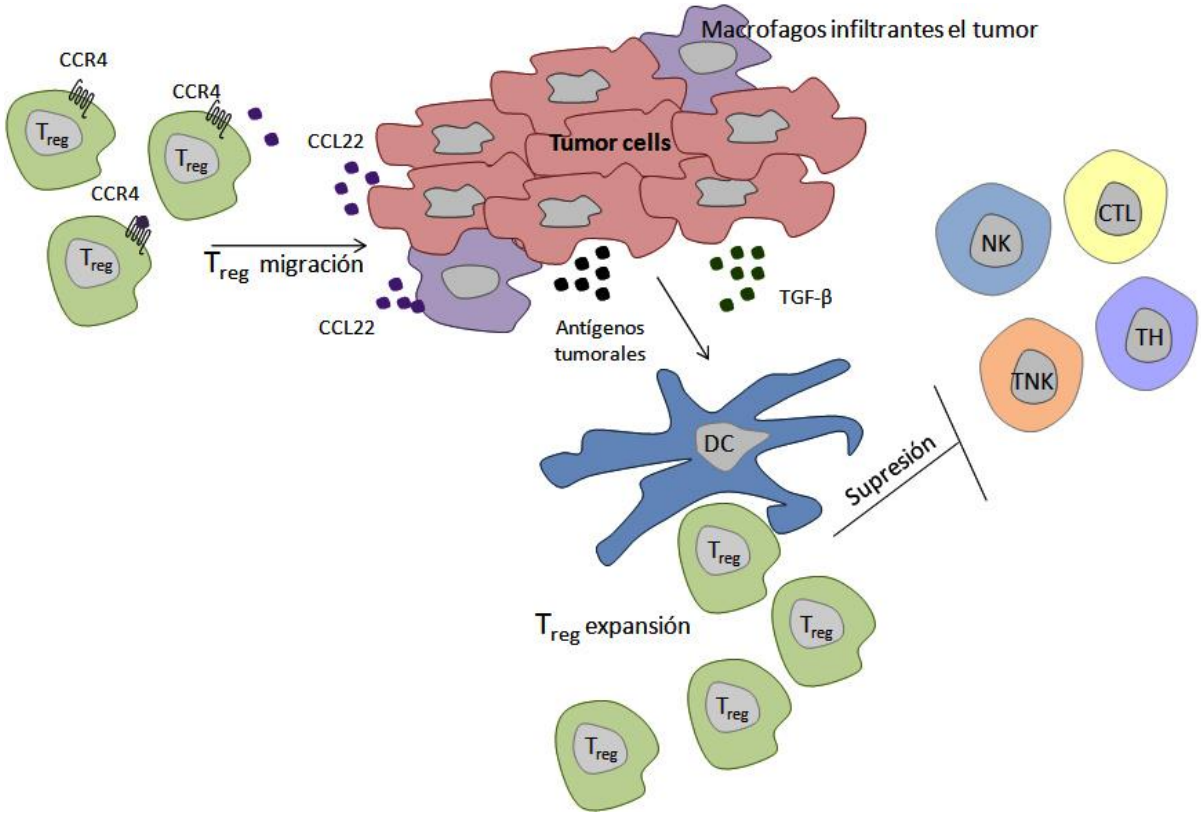


Figura 3. Supresión de la respuesta inmune tumoral mediada por las células T_{reg}
Tanto las células tumorales como los macrófagos asociados producirían la citoquina CCL22, responsable de la atracción de las células T_{reg} a través del receptor CCR4 y por tanto de su acumulación en el microambiente del tumor (migración de T_{reg}). Las células T_{reg} entonces recibirían señales de proliferación (expansión de iT_{reg}) a través de la presencia de antígenos propios asociados al tumor y de los altos niveles de TGF-β. Las DCs favorecerían aún más la expansión de células T_{reg}. El gran número de células T_{reg} presentes inhibiría a las células T natural killer (NTK), a los linfocitos T citotóxicos CD8+ (CTL) y a las células T CD4+ colaboradoras, lo que contribuiría a la progresión del tumor. (imagen modificada de Nishikawa H, Sakaguchi S: Regulatory T cells in tumor immunity. Int J Cancer 2010. 127 (4):759-67) (41)).

El aumento de las células T_{reg} en el microambiente tumoral podría ser debido a la gran proliferación y posterior muerte de las células tumorales, las cuales proporcionarían una gran cantidad de antígenos propios (39). Las células T_{reg} FOXP3+ también podrían originarse por la activación de células T CD4 "*naive*" o vírgenes tras las altas concentraciones de TGF-β secretado por las células tumorales o por las DCs presentes en el tumor (40).

Por último, las células T_{reg} podrían inhibir la actividad de células T_{eff}, lo que daría lugar a una tolerancia frente al tumor (Figura 3).

El efecto generado por las células T_{reg} en la inmunidad tumoral en el caso de los linfomas es poco conocido. Estudios recientes sugieren que la composición celular del microambiente tumoral, en concreto la cantidad de T_{reg} infiltrantes, pueden modificar de forma significativa la evolución clínica de las neoplasias hematológicas. Al contrario de lo observado en tumores sólidos, en los linfomas de Hodgkin clásicos (cHL) (42) y foliculares (FL) (43) la presencia de un gran número de células T_{reg} se correlaciona con una mejor respuesta terapéutica y una mayor supervivencia. Estos estudios sugieren que la presencia de células T_{reg} en linfomas podría contribuir a la supresión, no sólo de las células citotóxicas infiltrantes, sino también de las propias células malignas. Por tanto, la implicación de las células T_{reg} en estos tumores sería más compleja que en el caso de los tumores sólidos, por lo que se necesitan estudios adicionales.

4. Células T colaboradora del centro germinal (T_{FH})

4.1 Las células T_{FH} como un subtipo de células T_H

Las células T_{FH} son células moduladoras esenciales para la respuesta inmune. A pesar de que carecen de la capacidad de atacar directamente a los patógenos invasores, son las responsables de coordinar la respuesta de otros muchos tipos celulares.

Las células T_{FH} son un subtipo de células T CD4+ que se localizan dentro de los folículos linfoides (área B) de los órganos linfoides secundarios (ganglios linfáticos, bazo y placas de Peyer). Los linfocitos T_{FH} se caracterizan por la expresión del receptor CXCR5 (*C-C chemokine receptor type 5*), mediante el cual migran a los folículos linfoides de los tejidos anteriormente citados, donde colaboran en la diferenciación celular de los linfocitos B (9, 10). Estudios previos basados en análisis de perfiles de expresión génica han demostrado que las células T_H que expresan altos niveles de CXCR5 presentan un perfil de expresión génica notablemente diferente de las células T_{H1}, T_{H2}, T_{H17} y de las células T_{reg}, por lo que las células T_{FH} forman una subpoblación de células T CD4+ (44, 45). A diferencia de otros subgrupos de células T, la transcripción génica de las células T_{FH} se caracteriza por la expresión de CD4, CD10, CXCR5, BCL6 (B-cell lymphoma 6), CD40L y CD57 (también presentes en las células B y en otros tipos de células T), así como por la expresión de otras moléculas cuya presencia parece ser más restrictiva de este tipo celular (Figura 1). Entre las moléculas relevantes para que las células T_{FH} puedan desarrollar sus funciones de ayuda a las células B se encuentran ICOS (*inducible T-cell co-estimulador*), SAP (*signaling lymphocytic activation molecule*

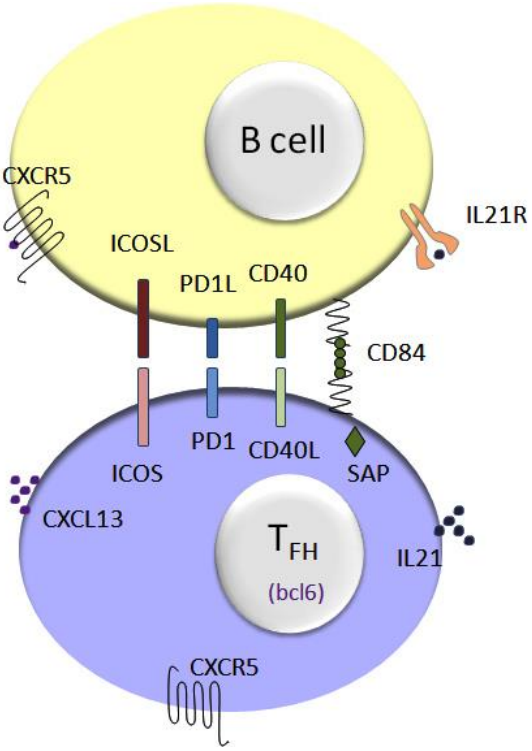


Figura 4. Interacción entre las células T_{FH} y los linfocitosB
Las interacciones entre las células TFH y las células B se encuentran mediadas por una serie de moléculas accesorias, como ICOS, PD1, CD40L y SAP en la célula TFH e ICOSL, PD1L, CD40 y la familia de receptores SLAM (CD84) en la célula B. Las células TFH también se caracterizan por la expresión de la proteína BCL6 y la producción de IL-21.

(SLAM)-associated protein) y PD-1 (*programmed cell death 1*) (Figura 4). Tanto el factor de transcripción BCL6 como la citoquina IL-21 tienen también un papel importante en la función y diferenciación de las células T_{FH}, siendo considerados actualmente marcadores específicos de este tipo celular.

4.2 El desarrollo de las células T_{FH}

El mecanismo de generación de las células T_{FH} no se conoce con claridad en la actualidad. Una de las posibles hipótesis se encuentra ilustrada en la Figura 5.

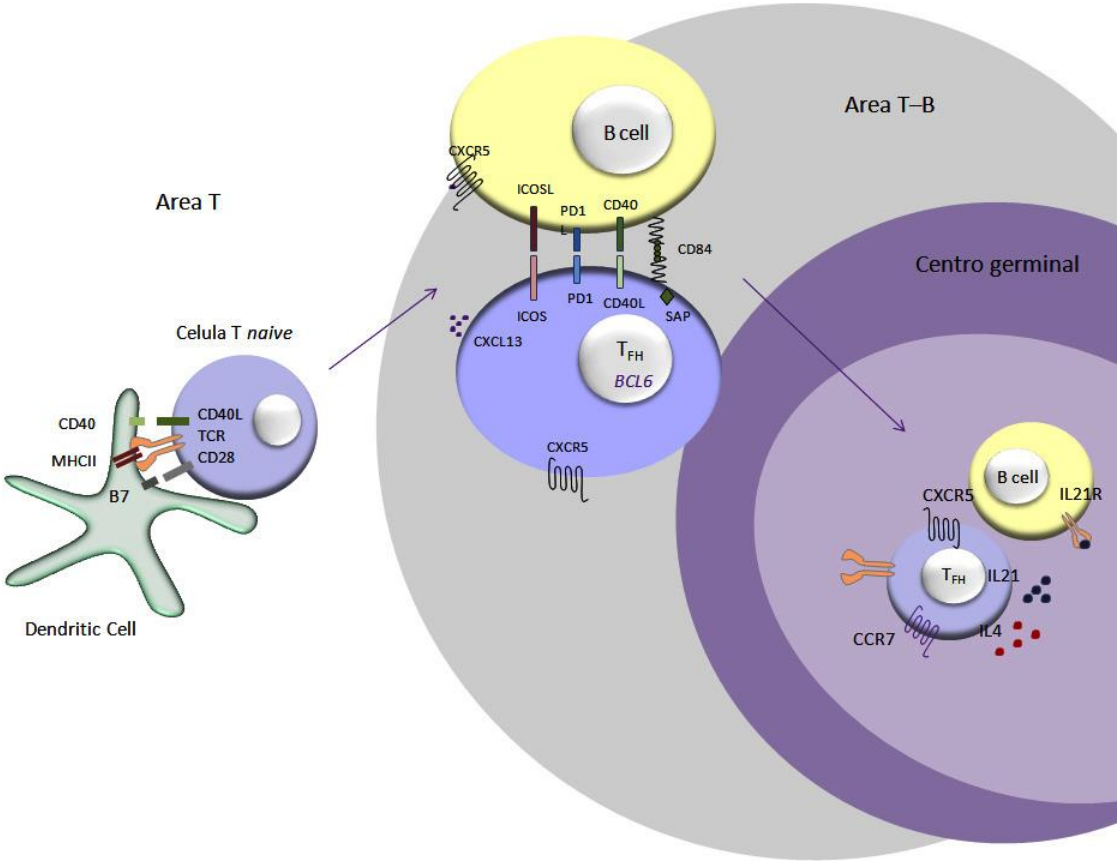


Figura 5. Generación de las células T_{FH}
En el área T de los órganos linfoides secundarios, las DCs activan a los linfocitos T CD4+ "*naive*" o vírgenes. Las señales de los receptores de las células T, junto con la co-estimulación proporcionada por las interacciones CD28/B7, CD40L/CD40, ICOS/ICOSL y PD1/PD1L son capaces de iniciar el programa de diferenciación de las células T_{FH}. El aumento en la expresión de CXCR5 ayuda a las células T_{FH} a localizar la región T-B, donde interactúan con las células B a través de las interacciones CD40L/CD40, ICOS/ICOSL, CD84/SAP y PD1/PD1L. Posteriormente, las células T_{FH} migran hacia el GC dónde colaboran en la diferenciación de los linfocitos B.

Una vez que las DCs reconocen al antígeno, éstas migran desde la periferia al área T de los tejidos linfoides, donde presentan al antígeno mediante el MHC II. Los linfocitos T CD4+ "*naive*" o vírgenes reconocen dicho antígeno mediante su receptor TCR específico, contactan con las DCs de forma estable y reciben señales de co-estimulación debido las interacciones CD28/B7, CD40L/CD40, ICOS/ICOSL y PD1/PD1L y por la activación de BCL6 (46). Los linfocitos T ya activados, disminuyen la expresión del receptor CCR7 (*chemokine (C-C motif) receptor 7*) y aumentan la expresión de CXCR5. Como consecuencia, estos linfocitos migran hacia el folículo, en concreto al área T y B, donde interaccionan con las células B para recibir una segunda cascada de señales de activación (25).

La importancia de las células B en el desarrollo de las células T_{FH} se basa en un estudio que ha demostrado que tanto la falta de células B como la ausencia de interacción B-T_{FH}, produce una ausencia de células T_{FH} (47). Los contactos permanentes entre las células B y T se encuentran mediados por el receptor TCR y el MHC II. Una vez más, una amplia gama de receptores de superficie celular como CD40/CD40L, ICOS/ICOSL, miembros de la familia SLAM y PD1/PD-L1 son cruciales para completar y mantener la diferenciación de las células T_{FH} (48).

Estas interacciones culminan con la secreción por parte de las células T_{FH} de citoquinas, en particular las IL-4 e IL-21, y la migración de las células T_{FH} hacia el GC de los folículos (49). Aquellas células T activadas que no logran obtener las señales necesarias no entrarán en los GC.

4.3 Las proteínas SAP y PD-1 en el desarrollo de células T_{FH}

Como ya ha sido mencionado, la familia de receptores de superficie SLAM juega un papel importante en la generación de las células T_{FH}. La relevancia de estas moléculas en las interacciones T-B fue descubierta a raíz de los estudios con pacientes de XLP (enfermedad inmune linfoproliferativa ligada al cromosoma X). Esta inmunodeficiencia está causada por mutaciones en el gen que codifica para la prot*eína SAP (SH2D1A),* molécula adaptadora citoplásmatica que interviene en la activación posterior de genes relacionados con la familia de receptores SLAM. Los pacientes con XLP, así como los ratones modificados genéticamente que carecen de expresión de la proteína SAP, exhiben una respuesta defectuosa de células B dependiente de células T (50, 51).

Estudios recientes han demostrado que la proteína SAP, que actúa después de CD84, es necesaria para regular la adhesión entre las células T y B. Por lo tanto, en ausencia de SAP o CD84, las células T CD4+ son incapaces de formar uniones permanentes con las células B, produciendo una disminución de las señales co-estimuladoras procedentes de las moléculas de superficie antes citadas y de las citoquinas que directamente influyen en la diferenciación y supervivencia de las células B (52).

Otra molécula expresada por las células T_{FH} es PD-1 (CD279). PD-1 y sus ligandos (PD-L1 y PD-L2) juegan un papel importante en la inhibición de la regulación de la homeostasis inmune e intervienen en el mantenimiento de la tolerancia periférica (53). La evidencia de que PD-1 suprime la activación de la respuesta inmune proviene de estudios con ratones deficientes para esta proteína, los cuales desarrollan enfermedades autoinmunes espontáneas tales como el lupus eritematoso sistémico, miocardiopatía dilatada, artritis reumatoide o diabetes mellitus tipo I, debido a la activación persistente y descontrolada de las células T (54).

Aunque la expresión de la proteína PD-1 es elevada en las células T_{FH}, se sabe poco acerca del papel que juega en su desarrollo y función. Una posible función podría ser generar una señal inhibitoria sobre las células T_{FH}, evitando la proliferación excesiva de células T CD4+ en el GC (55, 56). Por otro lado se postula que la inducción de PD-1 por las células T_{FH} dentro del GC promueve la supervivencia y la selección de células B con receptores de inmunoglobulina de alta afinidad (57).

4.4 Función de las células T_{FH}

Las células T_{FH} se diferencian de otras subpoblaciones de células supresoras por su localización en los GCs de los órganos linfoides y por su capacidad para favorecer la activación, expansión y diferenciación de los linfocitos B y la formación del GC (58).

El mecanismo de “ayuda” de las células T_{FH} a las células B que se encuentra mejor caracterizado es el producido a través del ligando de CD40 (CD40L), cuya expresión en este tipo celular es muy alta. El CD40L es un activador muy potente de las células B, capaz de inducir proliferación celular. En combinación con determinadas citoquinas puede promover el cambio de isotipo y la diferenciación de las células B (59). La importancia de esta molécula en la respuesta de las células B ha sido demostrada en

modelos murinos que carecen de CD40 o CD40L, los quales presentan una disminución de la respuesta immunitaria y una incapacidad para generar GCs y células B de memoria (60).

Otro mecanismo por el cual las células T_{FH} regulan la respuesta de las células B es a través de la secreción de citoquinas. Las células T_{FH} se caracterizan por la expresión de IL-21, citoquina capaz de modular la diferenciación y la proliferación de las células B (61). Recientemente se han identificado otra serie de citoquinas (IL-4, IL-10, IL-17 e IFN-γ) secretadas por las células T_{FH} y que juegan un papel importante en el cambio de isotipo y la producción de anticuerpos (62).

4.5 Implicación de las células T_{FH} en los linfomas

Está aceptado de forma universal que las células T_{FH} juegan un papel importante tanto en el microambiente del GC como en el desarrollo de las células B. Además, diferentes estudios han demostrado que las células T_{FH} también pueden estar implicadas en enfermedades autoinmunes si se acumulan de forma aberrante o se desregulan (63). Estudios recientes sugieren que las células T_{FH} pueden desempeñar un papel importante en la biología de los tumores y en particular, en las enfermedades linfoproliferativas (64).

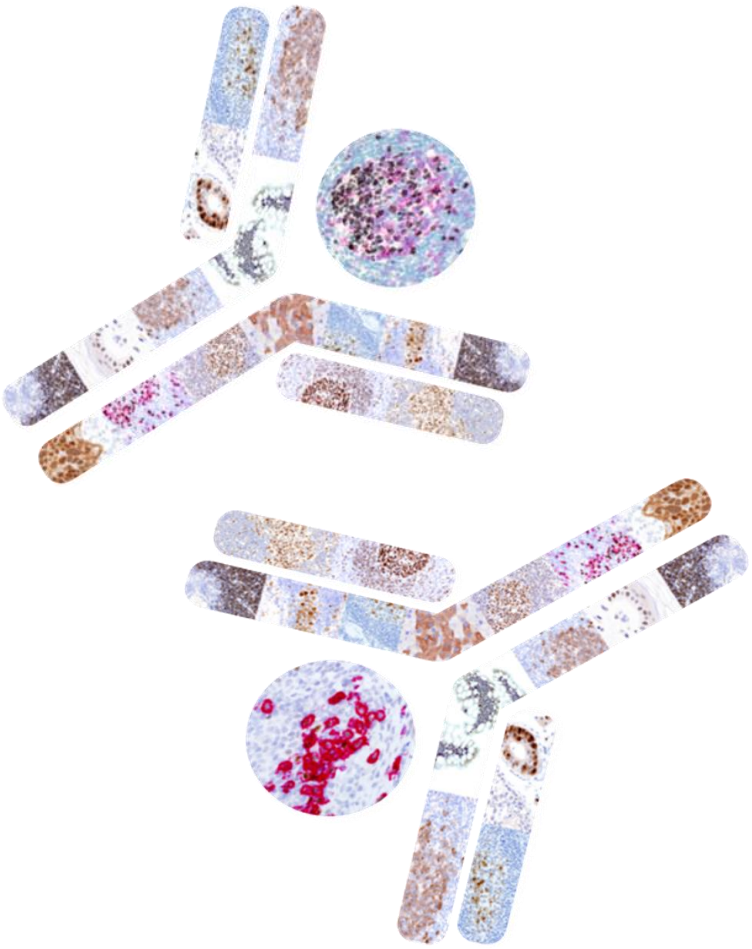
El microambiente del GC no es sólo un nicho esencial para la generación de la respuesta de las células B, sino que también se considera crítico para el desarrollo de la mayoría de las neoplasias linfoides humanas. Aunque la mayor parte de los linfomas que se originan de linfocitos del GC derivan de células B, se ha sugerido que algunos linfomas periféricos de células T, en concreto el angioimmunoblástico (AITL), podría tener su origen en las células T_{FH} del GC. Esta hipótesis ha sido confirmada con estudios de perfiles de expresión génica llevados a cabo en linfomas periféricos de células T, los cuales han demostrado que la célula T_{FH} representa la célula de origen del AILT, habiendo sido identificados PD-1 y CXCL13 como marcadores de esta subpoblación de células T (44).

Otros estudios han puesto de manifiesto que los marcadores de T_{FH} no son exclusivos del AITL, ya que también pueden estar presentes en otros tipos de linfoma de células T, concretamente en el linfoma cutáneo de célula T CD4+ pleomórfico de tamaño pequeño/mediano (CSTCL). Este hallazgo sugiere que no solo en el GC, sino también en

otras localizaciones podría tener lugar un proceso de estimulación de células B por parte de las T_{FH} (65).

En los últimos años, varios estudios han puesto de manifiesto la presencia de células T_{FH} en una gran variedad de microambientes de linfomas B, destacando la importancia de la identificación de este tipo celular como una herramienta adicional para el diagnóstico de linfomas (64). Además, se ha comprobado que la presencia de T_{FH} en el microambiente tumoral puede influir directamente en el desarrollo del tumor y podría ayudar a predecir tanto la respuesta a la terapia como la supervivencia (64). Un ejemplo lo constituye la presencia de células T_{FH} en el linfoma de Hodgkin de predominio linfocítico (NLPHL), un tipo de linfoma de crecimiento lento caracterizado por la presencia de una pequeña población de células B neoplásicas dentro de un fondo enriquecido de linfocitos e histiocitos. La distribución de células T_{FH} en este tipo de tumores no es aleatoria, ya que las células T_{FH} PD-1 positivas forman rosetas alrededor de las células B neoplásicas, donde es probable que participen en el crecimiento y la supervivencia del tumor (66).

El FL es una neoplasia derivada de los linfocitos B del GC, donde las células tumorales proliferan en los folículos en asociación estrecha con las células T_{FH} y las DCs foliculares (67). Un estudio reciente ha demostrado que el número de células PD1 positivas que infiltran el tumor es un factor predictivo de supervivencia en pacientes con FL, lo que sugiere que PD-1 es capaz de identificar una subpoblación importante de T_{FH} en el microambiente de este tipo de linfomas. Dicha subpoblación podría participar directamente en la modulación del comportamiento de las células tumorales y por tanto, influir en su evolución (68).



OBJECTIVES

The overall goal of the present thesis is to study and characterize the T_{reg} and T_{FH} cell subtypes in reactive and lymphomas human tissues using two novel monoclonal antibodies against FOXP3 (T_{reg} marker) and PD-1 (T_{FH} marker). This work also analyses the possible diagnostic and prognostic value of FOXP3 and PD-1 mAbs in a survey of human lymphomas, focusing in particular on T-cell lymphomas.

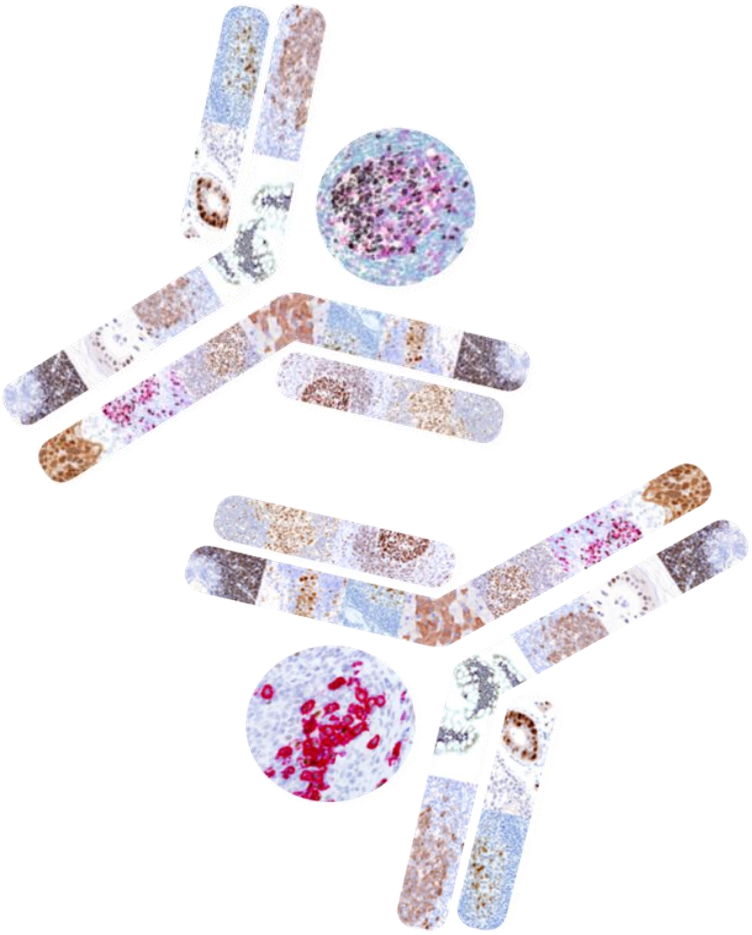
To achieve this overall goal we propose the following specific aims:

First project

- Production and characterisation of a novel FOXP3 mAb at single cell level.
- To establish the predictive value of the detection of FOXP3 protein in routine paraffin tissue sections in a large set of lymphomas to evaluate its possible use as a diagnostic biomarker and its correlation with patient outcome.
- To assess the presence of FOXP3+ T tumour-infiltrating cells in lymphomas and its correlation with patient outcome.

Second Project

- To study the distribution and phenotypic characteristics of PD-1 and SAP positive cells at single cell level by applying multi-immunolabelling techniques in lymphoid tissues.
- To assess the diagnostic relevance of PD-1 and SAP protein in a set of human lymphomas focusing in particular on AITL and other subtypes of T-cell lymphomas.
- To assess the diagnostic and prognostic potential of PD-1 as a marker of T_{FH} cells present in the tumour microenvironment of nodular lymphocyte-predominant Hodgkin lymphoma.



MATERIALS AND METHODS

Materials and Methods

1. Generation of FOXP3 and PD-1 monoclonal antibodies

1.1. Antigen preparation

1.1.1 Expression and purification of FOXP3 protein

The cDNA clone containing the FOXP3 cDNA (IMAGE: 5747723) was obtained from the MRC geneservice. A cDNA fragment encoding the full-length human FOXP3 protein was amplified by PCR using the High Fidelity PCR system (Roche, Germany) and the primers: Forward 5'-GACAAGGATCCGATGCCCAACCCCAGGCC-3' and reverse 5'-CAGCAAAACGAGCTCTCAGGGGCCTCCC-3', both primers contain BamHI and XhoI restriction sites, to facilitate directional cloning. The amplified product was inserted between BamHI and XhoI sites of the pENTR2B (Invitrogen, USA), using restriction enzyme digestion and ligase-mediated cloning. The resulting recombinant plasmid pENTR-FOXP3 was fully sequenced. Subsequently, the insert was transferred by means of Gateway technology into the E. coli destination vector pDEST15 (Invitrogen, USA) using the LR clonase enzyme mix (Invitrogen, USA), transformed into DH5α-competent cells and gene insertion was confirmed by PCR. The expression plasmid was named pDEST15-FOXP3 and was used to transform BL21 (DE3) pLysS competent cells (Novagen, Germany). A single colony was grown overnight at 37ºC in LB medium supplemented with 100 µg/ml ampicillin and 35 µg/ml chroramphenicol. After overnight growth, the cells were diluted 100-fold in fresh medium and grown at 37ºC to mid-log phase (A600= 0.5-0.7), where induction was initiated by the addition of 0.4 mM IPTG (Roche, Germany). After 3 h induction, cells were harvested by centrifugation and resuspended in 0.02 culture volumes of PBS. Three freeze-thaw cycles followed by sonication (three bursts of 30 seconds each) on ice were used to disrupt the cells and the soluble and insoluble fractions were separated by centrifugation. The levels of expression and solubility were analysed using Coomassie-stained SDS-PAGE and immunoblotting as described below.

Recombinant GST-FOXP3 contained in the soluble fraction of induced cells was purified by affinity chromatography using a GSTrap column (GE Healthcare, USA) on an ÄKTA Prime (GE Healthcare, USA). The binding buffer was 20 mM sodium phosphate pH 7.3 with 0.15 M NaCl and the elution buffer was 50 mM Tris-HCl pH 8.0 with 10 mM

reduced glutathione. After elution by means of a step gradient from 0% to 100% of elution buffer, fractions were monitored at 280 nm and those containing GST-FOXP3 were pooled and dialyzed against PBS.

1.1.2 YT cell line was used as immunogen for the production of PD-1 monoclonal antibody

A human T/NK cell line called YT (DSMZ, Germany), expressing high level of PD-1 protein, was used as a source of antigen. All cells were cultured in RPMI medium (Gibco, Germany) containing 10% fetal calf serum (FCS) (Gibco, Germany), 4 mM glutamine, 10 U/ml penicillin and 100 µg/ml streptomycin (Boehringer, Germany).

1.2. Immunization

Two female Balb/c mice were immunized intraperitoneally (i.p.) with 100ug of purified GST-FOXP3 protein in phosphate-buffered saline (PBS) with 1:1 complete Freund's adjuvant (Chemicon, USA). For the production of PD-1 antibody two female Balb/c mice were immunized i.p. with $2x10^6$ YT cells in PBS without Freund's adjuvant. At day 14, the mice were injected i.p. with additional 100 µg of purified GST-FOXP3 without Freund's adjuvant (Chemicon, USA) and with $2x10^6$ YT cells respectively. At Day 28, the mice were injected i.p. with an additional boost of 150ug of purified GST-FOXP3 protein and in case of PD-1 mice with a boost of $2x10^6$ YT cells. Mice were bled for serum titers determination on day 38. In case of FOXP3, mice received a final boost of 150ug of GST-FOXP3 protein in PBS, i.p., 3 days prior to fusion. In case of PD-1, mice received a final boost of $2x10^6$ YT cells i.p.

1.3 Fusion procedure

1.3.1. Culture myeloma cells

One week before fusion, we proceed to the expansion of NS1/Ag4-1 (NS-1) myeloma cell line (the fusion partner cell line). NS-1 cell line was cultured in RPMI medium (Sigma-Aldrich, USA) containing 10% FCS (Gibco, Germany), 4 mM glutamine, 10 U/ml penicillin and 100 µg/ml streptomycin (Boehringer, Germany). One day before the

fusion, we moved the cells into fresh medium with a final concentration of $5x10^5$ cells/ml. The cell viability at the time of cell fusion was greater than 95%.

1.3.2 Standard fusion procedure

Three days after the final booster the mouse was sacrificed. We aseptically removed the spleen from the selected mouse, we placed it in a 60-mm-diameter Petri dish with 5 ml of complete medium (RPMI medium containing 10% of FCS, 4 mM glutamine, 10 U/ml penicillin and 100 µg/ml streptomycin), and we carefully teased it apart. The cell suspension was then transferred to 10ml falcon tube. The spleen cells were centrifuged (1000rpm for 10 min) and the cell pellet was resuspended with 5 ml of RPMI medium without serum. At the same time the NS-1 myeloma cell line was washed with 5ml of RPMI medium without serum and centrifuged (1000rpm for 10 min). Both cell pellets were resuspended in a pre warmed medium without serum and combined. The cell suspension was centrifuged (1000rpm for 5 min) and the medium was then carefully removed. The pellet was disaggregated by gently tapping the bottom of the tube. 1.2 ml of warmed (37°C) 50% polyethylene glycol 1500 (Roche, USA) was slowly added, drop by drop, for 1 minute with gentle mixing. 2 and 4 mls of warm (37^oC) medium without FCS were added over a period of 2 and 4 minutes, again gently swirling the tube. A final volume of 8ml of medium with 10% FCS was finally added. The cells suspension was then centrifuged (1000rpm for 5 min).

The cell pellet was carefully resuspended in 350 ml of complete RPMI media and plated in 14 plates (24-well tissue culture plates) (Thermo Fisher, USA). The plates were then placed in a 5% CO2/air incubator at 37°C. The day after, 1 ml of complete media with 2xHAT (hypoxanthine, aminopterin and tymidine) (Gibco, Germany) was added in each well. Clones were visible by microscopy at about day four and by eye starting at day 7. After 10 days the plates were inspected for growth and supernatants were collected for ELISA testing.

1.3.3 Single-cell cloning by limiting dilution

Because more than one hybridoma colony might be growing in the same plate well, cells that give a positive result in the screening assays must be isolated. A general

procedure for cloning individual hybridomas consists in the single cell cloning technique.

The hybridoma cells were healthy and rapidly growing at the time of cloning. A tube with 10 ml of supplemented medium (RPMI medium containing 10% FCS, 4 mM glutamine, 10 U/ml penicillin, 100µg/ml streptomycin and 2% condimed (Roche, USA) was prepared. 10 µl media containing hybridoma cellsfrom the hybridoma flask was added to the 10 ml tube. After counting the cells present in the 10 ml tube, we worked out the volume of medium that was containing 96 cells. The correct volume was added in 16 ml of supplemented medium and, using a multiwell pipettor (Thermo Fisher, USA), 200 µl of the 16ml medium was added in a 98 well culture plate. The 98 well plates were then placed in a 5% CO2/air incubator at 37°C. Clones appeared in around 7 days and they were ready to be screened after 10 days. Supernatant coming from the wells containing a single colony were tested by ELISA or imminohistochemisty in tranfected cells. The clones that secrete the desired antibody were expanded and several aliquots of them were frozen before proceeding to large-scale antibody production.

2. ELISA (enzyme-linked immuno-absorbant assay)

ELISA testing was performed to screen fusion supernatant since is the most suitable screening assay for soluble antigen. Ninety-six-well microtiter plates (Nunc, Denmark) were coated for 1 hr at 37ºC with 50µl/well of the proper antigen (diluted 5µg/ml in distilled water). The plates were then washed three times with PBS/Tween and 200 µl/well of PBS plus 2% of dry milk powder was added. The plates were left overnight in a cold room. One day after the plates were washed 3 times in PBS/Tween and 50µl of each supernatant that you wished to test were added to the plates and incubated for 1 hr at 37ºC. We also added to the ELISA plate the mouse serum coming from the same mouse, diluted 1:100 and 1:200 in PBS as a positive control. The negative control used was the PBS alone. The plates were then washed three times with PBS/Tween and three times with distilled water. 100 µl of substrate buffer (Roche ABTS Solution, USA) were then added. After around 15 minutes the positives clones were visible and selected.

3. Eukaryotic expression of proteins in COS-1 and HEK293T cells

To confirm that our mAbs were specifically reactive with the FOXP3 protein (Forkhead box protein P3 (NP-054728.2)), their reactivity was tested on COS-1 cells (fibroblast-like cell line derived from monkey kidney tissue, ATCC, USA) expressing FOXP3 and on COS-1 cells expressing the closely related FOXP1, FOXP2 and FOXP4 proteins. The Flag-tagged FOXP3 cDNA was kindly provided by Dr Mary Brunkow, Celltech.

On the other and to confirm that the monoclonal antibody NAT recognized human PD-1 protein (Homo sapiens programmed cell death 1 (NP_005009.2)), a pCMV6-XL5-PD-1 vector (Origene, USA) expressing human PD-1 was transfected into HEK293T cells (Human embryonal kidney cell line, ATCC, USA).

Plasmid DNAs were prepared for transfection using the Plasmid Midi Kit according to the manufacturer's instructions (Genomed, Germany). COS-1 cells were respectively transfected with pcDNA4/HisMax/ FOXP2, FOXP3, pcDNA4/HisMax/FOXP4, pcDNA4/HisMax (kindly provided by Dr Mary Brunkow, Celltech) alone or pAB195 (FOXP1) using Fugene 6 transfection reagent, following the protocol described by the manufacturer (Roche, USA). The HEK293T cells were tranfected with a pCMV6-XL5-PD-1 vector using the same protocol used for FOXP family vectors.

Cell pellets were snap-frozen and stored at -70^{o}C whereas cytocentrifuge preparations were made for immunocytochemical staining and stored at -20ºC. Paraffin-embedded cell pellets were prepared by fixing transfectants for 48 h in neutral buffered formalin (10% formalin in PBS), before centrifugation into 2% agar in neutral buffered formalin; the agar pellet was then embedded in paraffin and sectioned as for tissues.

4. Immunohistochemistry in cytospin of trasfected COS-1 or HEK293T cells

Since screening for the presence of specific antibodies by ELISA may not able to detect antibodies that recognize conformational epitopes of interest we also performed a secondary screening technique on the positive clones selected by ELISA. Immuhohistochemistry in transfected COS-1 and HEK293T cell expressing the protein of interest was performed as follow: A suspension of COS-1 or HEK293T tranfected cells containing 0.5x10^{6} cells/ml was prepared. We assembled the cytospin slide holder

(Thermo Fisher, USA) using an ethanol-cleaned slide and a cytospin filter. We transferred 80 to 150 µl of cell suspension into the cytospin chamber and centrifuge (1000rpm for 5 min) at room temperature. The slides obtained were dried 24 hr at room temperature. Cytospin preparation were incubated with 100µl the supernatnat for 30 min at room temperature. The slides were then incubated with HRPconjugated goat anti-mouse-Ig (diluted 1:100 in PBS) (Dako, Denmark). The peroxidase reaction was developed using 3, 3'-Diaminobenzidine (DAB) (Dako, Denmark) for 5 min and washed with distilled water.

5. Antibodies

The antibodies used in the thesis are summarized in the Table 1.

Table 1. Antibodies used in FOXP3 and PD-1 study

Anti-	Clone name	Source
BCL6	GI191E/A8	CNIO Monoclonal Antibody Unit, Madrid, Spain
CD3	A0452	Dako, Glostrup, Denmark
CD4	MT310	Dako, Glostrup, Denmark
CD8	C8/144B	Dako, Glostrup, Denmark
CD10	NCL-L-CD10-270	Novocastra Laboratories, Newcastle, UK
CD20	L26	Dako, Glostrup, Denmark
CD25	4C9	Novocastra, Newcastle upon Tyne, UK
CD57	B321 (NK-1)	Abcam, Cambridge, UK
CXCL13	53610	R&D Systems, Minneapolis, USA
PD-1	NAT	CNIO Monoclonal Antibody Unit, Madrid, Spain
FLAG	F3165	Sigma-Aldrich, USA
FOXP1	JC12	Abcam, Cambridge, UK
FOXP3	Goat polyclonal	Abcam, Cambridge, UK
GAPDH	FF26	CNIO Monoclonal Antibody Unit, Madrid, Spain
SAP	FL-128	Santa Cruz Biotechnology, Ca, USA
Xpress	R910-25	Invitrogen, Oregon, USA

6. Patient samples

6.1 Patient samples used in the FOXP3 project

Lymph node biopsies from 23 patients with adult T-cell leukaemia/lymphoma (ATLL) were provided by Dr K Ohshima from the Department of Pathology, School of

Medicine, Fukuoka University, Fukuoka, Japan and two cases where obtained from the tissue archives of the CNIO Tumour Bank. HTLV-1 provirus integration was confirmed in all ATLL samples by ELISA and Southern-blot techniques.

A large series of samples representing T- and B-cell neoplasms, including T-cell lymphoblastic lymphoma (4), peripheral T-cell lymphoma (20), angioimmunoblastic T-cell lymphoma (5), T cell/natural killer cell lymphoma (5), anaplastic large cell lymphoma (9), mycosis fungoides (15), B-cell lymphocytic leukaemia/lymphoma (14), mantle cell lymphoma (14), marginal zone B-cell lymphoma (12), diffuse large B-cell lymphoma (17), follicular lymphoma (17) and Burkitt's lymphoma (15) were used in this study. Informed consent was obtained from all patients included in the study under the supervision of the local ethical committees. Reactive (non-neoplastic) lymphoid tissues (five tonsillectomy specimens, five lymph nodes, five spleens and five thymus) were obtained from the CNIO Tumour Bank. Frozen sections of spleen and lymph node were also obtained from normal mice (Balb/c or B6 strains).

6.2 Patient samples used in the PD-1 project

Biopsy samples (fresh and formalin-fixed paraffin-embedded tissues) were obtained from the tissue archives of the CNIO Tumor Bank, from the files of the Histopathology Department ,John Radcliffe Hospital, from the Institute of Pathology, University of Kiel and from the Department of Pathology, University Clinic, Frankfurt. The material investigated comprised sections of either whole tissue blocks or tissue-arrays, prepared as illustrated above. The diagnosis of angioimmunoblastic T cell lymphoma was based on a combination of morphologic features (e.g. "clear cell" neoplastic cell morphology, expanded follicular dendritic cell meshworks and vascular proliferation) and phenotype (e.g. expression by tumor cells of CD3 and CD4 accompanied by CD10 and/or BCL6).

One hundred fifty-two cases previously diagnosed as nodular sclerosis Hodgkin lymphoma (NSHL), mixed cellularity classical Hodgkin lymphoma (MCHL), lymphocyte-rich classical Hodgkin lymphoma (LRCHL), nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) and T cell /histiocyte-rich B cell lymphoma (T/HRBCL) were obtained from the files of the Molecular Pathology Programme of the CNIO. The cases were classified into 7 diagnostic groups: NSHL (n=43), MCHL (n=14), LRCHL (n=13),

NLPHL (n=58), NLPHL with diffuse areas (n=7), T/HRBCL (n=12) and NLPHL versus T/HRBCL (n=5). Briefly, NLPHL tumors were considered to be those with scattered CD30-negative tumoral cells within follicular dendritic cell meshworks accompanied by follicular T-cell rosettes surrounding the tumoral cells. Cases featuring diffuse areas with frequent tumoral cells, lacking follicular dendritic cell meshworks, in association with typical nodular zones of NLPHL, were classified as NLPHL with diffuse areas. T/HRBCL cases were characterized by diffuse areas with scattered CD20-positive tumoral cells, surrounded by numerous T cells and histiocytes without follicular dendritic cell meshworks. Five cases could not be classified either as NLPHL, NLPHL with diffuse areas, or T/HRBC and so were assigned to a separate group named as NLPHL versus T/HRBCL. All samples were reviewed by at least two pathologists, and in each case diagnosis had been made on the basis of conventional histologic and immunohistologic examination according to the criteria of the World Health Organization (WHO) classification (1).

7. Tissue microarrays preparation

Tissue microarrays (TMA) were built using tissue cores from formalin-fixed paraffin embedded tumours. Hematoxylin and eosin (H&E) stained slides were reviewed, and areas containing tumour tissue were marked on both the slides and corresponding paraffin blocks for tissue microarray construction. Microarray blocks were constructed using a manual arrayer (Beecher Instruments, USA) as previously described (69). Briefly, the instrument was used to create holes in a recipient block with defined array coordinates. A solid stylet was used to transfer the tissue cores into the recipient block. Two 0.6-mm-diameter tissue cores were taken from each case. Several composite high-density TMA blocks were designed, and serial 4-µm sections were cut with a microtome (Leica, Germany) and transferred to poly-l-lysine coated slides (Thermo Fisher, USA). One section from each tissue array block was stained with H&E. The remaining sections were stored at room temperature.

8. Immunohistochenistry

8.1 Immunohistochemistry in frozen tissue sections

Frozen tonsil tissue sections were incubated for 30 min with primary Ab, washed in PBS and incubated with either HRPconjugated goat anti-mouse-Ig (diluted 1:100 in PBS)(Dako, Denmark) or HRP conjugated rabbit anti-goat-Ig (diluted 1:100 in PBS)(Dako, Denmark). The peroxidase reaction was developed using DAB (Dako, Denmark) for 5 min and washed with distilled water. Murine tissue sections were fixed in acetone, blocked with 10% normal FCS, incubated with each mAb followed by anti-mouse-Texas-red (diluted 1:100)(BD Bioscience, USA) and anti-CD4-FITC (diluted 1:75) (BD Bioscience, USA).

8.2 Immunohistochemistry in paraffin tissue sections

Immunohistochemistry on paraffin sections was performed using the pressure-cooking method of antigen retrieval (2 min, citrate pH 6.5 or Tris-EDTA buffer). Before staining, endogenous peroxidase was blocked; the slides were incubated for 40 min with the primary antibody and washed with PBS. Immunodetection was performed with biotinylated anti-mouse secondary antibodies (25 min), followed by peroxidase-labelled streptavidin (LSAB- Dako, Denmark) (25 min) and DAB chromogen as substrate. All immunostaining was performed using the Techmate 500 automatic immunostaining device and reagents supplied by DakoCytomation (Dako, Denmark).

8.3. Double immunoenzymatic staining

In the first reaction, immunostaining was performed using the EnVision peroxidase kit and diaminobenzidine (DAB) chromogen-substrate (Dako K5507, Dako, Denmark). In the second reaction, immunostaining was performed using the alkaline phosphatase kit (Dako K5355, Dako, Denmark) and the chromogen provided with the kit manufacture instruction.

9. Immunofluorescence

Double immunostaining was performed by incubating paraffin sections following antigen retrieval for one hour at room temperature in a humid chamber with a mixture of the two primary antibodies diluted in PBS + 10% FCS (Life Technologies, USA). Slides were then washed three times in PBS containing 0.5% Tween 20 for periods of 5 minutes. Slides were subsequently incubated for one hour with fluorochrome-conjugated antibodies specific for different Ig isotypes (Molecular Probes, The Netherlands) (diluted in PBS 1:200) in a humid chamber in the dark. Slides were washed three times subsequently in PBS containing 0.5% Tween 20 for 5 minute periods. Following washing, sections were mounted in antifading mounting medium (Qbiogene, France) containing DAPI (Molecular Probes, The Netherlands). Slides were examined on a Nikon E800 Eclipse fluorescence microscope (Nikon, Kingston-upon-Thames, UK) equipped for epifluorescence, and images were captured with an Axiocam chargecoupled device (CCD) camera (Zeiss, Germany) and Axiovision software (Imaging Associates, UK), and adjusted using Photoshop software (Adobe, San Jose, USA).

10. Immunoperoxidase staining, combined with single or double immunofluorescence

Slides were immunostained for corresponding antibody by the two-stage peroxidase-based EnVision technique, washed in PBS for up to 5 minutes. Haematoxylin staining was omitted .The slides were then incubated in FCS for up to 10 and incubated for 45 minutes at room temperature with a the corrisponding antibodies that were either from different species or of differing immunoglobulin isotype/subclass. Sections were washed in PBS for up to 5 minutes and then incubated in the dark for 45 minutes with secondary antibodies (specific for species, isotypes, or subclass) and labelled with contrasting green and red fluorochromes (Alexa Fluor 488 and Alexa Fluor 594, dilution 1:100) (Molecular Probes, The Netherlands). The slides were washed in PBS for up to 5 minutes, mounted in fluorescent mounting medium (Dako, Denmark) containing 1 µg/ml DAPI (4,6-diamidino-2-phenyllindole).

Slides were examined on a Nikon E800 Eclipse fluorescence microscope (Nikon, UK) equipped for epifluorescence, and images were captured with an Axiocam chargecoupled device (CCD) camera (Zeiss, Germany) and Axiovision software (Imaging Associates, UK), and adjusted using Photoshop software (Adobe, USA).

11. Western Blotting

To extract total protein, cells were lysed in a buffer containing 50 mM Tris (tris (hydroxymethyl) aminomethane)-HCl, pH 7.5, 150 mM NaCl, 1% Igepal (Sigma, USA) and protease inhibitors (Roche, Germany). Lysates were incubated in a cool room on a rotary shaker for 1 hour and cleared by centrifugation.

The total lysates of each cell line were denatured by heating in Laemmli sample buffer, resolved in a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and transferred onto nitrocellulose membranes for 2 hours. Membranes were incubated overnight with blocking solution (5% milk in PBS) and immunoblotted for 1 hour at room temperature with the corresponding antibody (neat supernatant) and GAPDH monoclonal antibody (1:10) (CNIO, Spain), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000) (DAKO, Denmark). Finally, the blots were visualized using the ECL detection system (Amersham Biosciences, UK) in accordance with the supplier's instructions.

12. T cell clones and activation

The CD8+ clones, 2D10 and 3F6, were generated by tetramer staining and FACS cloning and maintained as described previously (70). The CD4+ clone TB1 generated from a patient with a thymoma, as described previously (71), was kindly provided by Professor Nick Willcox (Oxford, UK). The cells were maintained in culture in RPMI-1640 containing 10% fetal calf serum at 37ºC in 5%CO2. Cells were activated, within 10–14 days, by stimulation with 20 lg/ ml phytohemagglutinin (Sigma-Aldrich, USA) and irradiated allogeneic peripheral blood lymphocytes (50 Gy). Cells were considered to be resting after more than 21 days post-stimulation.

13. Cell isolation and immunofluorescent labeling of sorted cells

Human mononuclear cells were isolated from fresh blood by Lymphoprep (Nycomed, Oslo) gradient centrifugation. The CD4+ cells were obtained by negatively sorting the mononuclear cells with CD8 and CD14 magnetic beads (Miltenyi Biotech, Germany). These were then positively sorted for CD4+CD25+ cells using CD25 magnetic beads (Miltenyi Biotec, Germany). In some instances, CD4+CD25+ cells were isolated using the CD4+CD25+ regulatory isolation kit according to the manufacturer's instructions (Miltenyi Biotech, Germany). MACS-sorted CD4+CD25+ and CD4+CD25– cells isolated from human peripheral blood were spotted onto slides at 5 x 10^4/spot. Slides were air-dried and then frozen at -20ºC. Cells were fixed in pre-chilled acetone (-20ºC) for 5 min and blocked with 10% normal goat serum. Primary Ab (clone 221D/D3) was added overnight at 4ºC, followed by goat anti-mouse-AlexaFluor-488 (1:500) (Molecular Probes, The Netherlands). Cytocentrifuge preparations of T cells purified for the suppression assay were air-dried overnight, fixed in 50:50 acetone: methanol for 60 sec at room temperature, rinsed in PBS, then incubated with primary mAb (clone 236A/E7) for 50 min. Following a 5-min wash in PBS the secondary Ab (goat anti-mouse-IgG1-AlexaFluor-546, Molecular Probes) diluted 1:400, was applied for 30 min.

14. FACS staining of FOXP3-transfected COS-1 cells

COS-1 cells were permeabilised with FACS Perm 2 solution (BD Biosciences, USA). Cells were stained with each FOXP3 mAb and wiyh a secondary goat-anti-mouse-PE (Southern Biotech, USA). They were analyzed using FACS Calibur (BD Biosciences, USA) and CELLQuest software.

15. FACS methodology for detecting endogenous FOXP3

For FACS analysis, 5 x 10^5 cells were fixed in 1 ml of PBS with1% paraformaldehyde and 0.05% Tween-20 overnight at 4ºC. Cells were treated twice with 0.5 ml of DNAse at 100 Kunitz/ml according to the manufacturer's instructions (Sigma-Aldrich, USA). Staining steps were performed at room temperature for one hour. Cells were incubated with mouse anti-human-FOXP3 (clone150D/E4), washed with FACS buffer (PBS, 3.00% fetal calf serum, 0.50% Tween-20 and 0.05% azide). FOXP3 Ab binding was

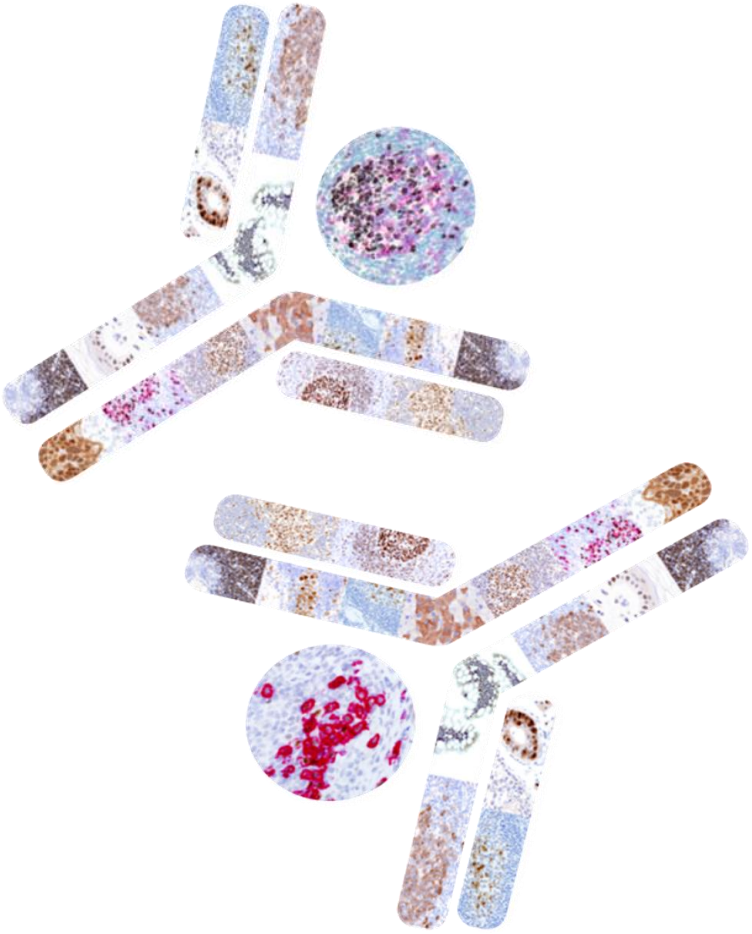
detected using Alexa Fluor-488J goat anti-mouse-IgG (Molecular Probes, USA) and washed as above. Cell surface staining was then performed using the mAb Cy-Chrome-anti-human-CD4 (Pharmingen) and PE-anti-human-CD25 (Miltenyi Biotec, Germany) for 20 min at room temperature followed by washing in PBS/BSA. Cells were analyzed using a FACSCaliburTM with CELLQuestTM software (Becton Dickinson, USA)

16. Cell proliferation assay

Cells were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 5% human AB serum, 2 mM L-glutamine (Gibco/Invitrogen, USA), 100 U/lg/ml penicillin/streptomycin (Gibco/Invitrogen, USA), 0.5 mM sodium pyruvate (Gibco/Invitrogen, USA) and 0.05 mM nonessential amino acids (Gibco/Invitrogen, USA) in 96-well plates (NalgeNunc, USA). Plate-bound anti-CD3 (clone UCHT1) and soluble anti-CD28 (clone 28.2) were purchased from Pharmingen (BD Biosciences Pharmingen). The CD4+CD25- responder cells were used at 5 x 10^4/well and a variable number of CD4+CD25+ regulatory cells were added. 3H-thymidine at 0.5 µlCi per well was added for the final 16 h of a 5-day assay.

17. Statistical analysis

Overall survival (OS) was calculated from the date of pathological diagnosis to death or to the last date of follow up. Actuarial survival curves, in terms of OS, were estimated by the Kaplan-Meier method and statistical significance of difference between FOXP3-positive and -negative cases were evaluated using the log-rank test. Median OS was also compared with a one-factor ANOVA method. A P-value of 0.05 or less was considered statistically significant. The SPSS software package (SPSS Inc., Chicago, IL; 1999) was used for the analyses.



RESULTS

1. First project: Regulatory T cells

1.1 The Abcam goat polyclonal FOXP3 Ab labels the human FOXP3 protein in frozen but not routinely fixed tissues

The commercially available Abcam goat polyclonal FOXP3 Ab was evaluated as a reagent to detect the FOXP3 protein in human tissues. Immunohistochemistry confirmed that the Ab recognized the human flag tagged FOXP3 protein expressed in COS-1 cell transfectants and stained scattered interfollicular cells in tonsil (Figure 1). However, we were unable to detect the FOXP3 protein in routinely fixed paraffin-embedded tissues with this reagent and thus additional reagents were necessary to investigate archival biopsy material.

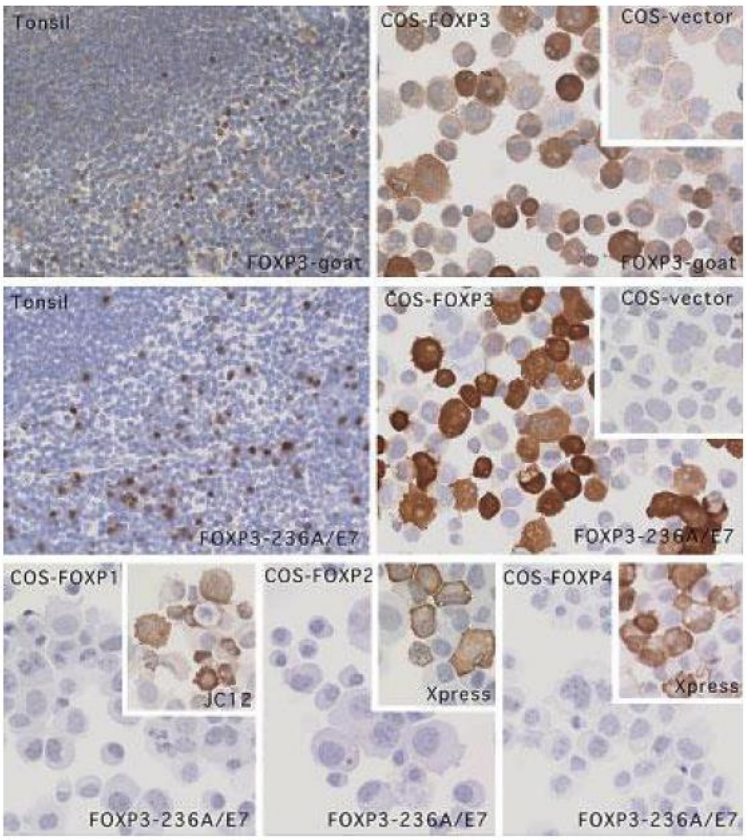


Figure 1. Immunolabeling of tonsil and FOXP transfectants. The top and middle rows show immunoperoxidase labeling with the commercial goat polyclonal Ab or the 236A/E7 mouse FOXP3 mAb, respectively, on tonsil and FOXP3-COS-1 transfectants. The bottom row shows the lack of staining with the 236A/E7 FOXP3 mAb on COS-1 cells transfected with the related FOXP proteins. The insets at top right of the bottom row confirm the expression of these recombinant FOXP proteins using the indicated Ab.

1.2 Generation of novel FOXP3 mAbs

The hybridoma fusion generated seven FOXP3 mAbs (86D/D6, 150D/E4, 157B/F4, 206D/B1, 221D/D3, 236A,E7 and 259D/C7) that showed a similar staining pattern, of scattered inter follicular cells on frozen tonsil, to that observed with the polyclonal FOXP3 Ab (Figure 1). We observed more FOXP3+ cells using our FOXP3 mAbs, which showed a stronger reactivity than was obtained with the polyclonal Ab. The reactivity of these FOXP3 mAbs was further investigated.

1.3 The mAbs specifically recognize FOXP3 and not other FOXP proteins

To confirm that the new mAbs recognized the human FOXP3 protein, expressed in COS-1 cells, was tested by immunohistochemistry on frozen cytospin preparations. Labeling with the anti-FLAG mAb confirmed the efficiency of transfection and the subcellular distribution of the recombinant FOXP3 protein. Cytoplasmic expression of proteins that are normally nuclear is commonly observed when cells are transfected using the described Fugene protocol and harvested after 24 h. All of the 7 mAbs specifically recognized the human FOXP3 protein by immunohistochemistry. There is significant sequence similarity between the four members of the FOXP family, FOXP1–4, particularly within the DNA binding forkhead domain. As a full-length FOXP3 protein was used as an antigen it was important to ensure the specificity of the mAbs for FOXP3 and exclude the possibility of their cross-reactivity with other FOXP proteins. Expression of the FOXP1, FOXP2 and FOXP4 proteins in transfected cells was confirmed using the JC12 mAb to label FOXP1 and the anti-Xpress antibody to detect epitope-tagged FOXP2 and FOXP4 (Figure 1). All the FOXP3 mAbs specifically labeled the COS-1 cells expressing FOXP3 and not those expressing the other FOXP proteins.

We have investigated a FOXP3 murine mAb, hFOXY (Cat No 14-5779, eBioscience, USA) that is commercially available and compared its reactivity on tonsil with that obtained with our 236A/E7 FOXP3 mAb (data not shown). On frozen tonsil sections hFOXY stained scattered interfollicular cells at a dilution of 1:25. At this dilution some background staining was observed and there was a noticeably smaller population of nuclear positive cells than was stained with our FOXP3 mAb 236A/E7. Staining of COS-1 cell transfectants confirmed that hFOXY detected the FOXP3 protein and not the related FOXP1, FOXP2 or FOXP4 proteins. Staining of paraffin-embedded COS-1 cell

transfectants indicated that hFOXY recognizes a formalin-resistant epitope on FOXP3 and no cross-reactivity was observed under these conditions with other FOXP proteins. Thus hFOXY is suitable for detecting FOXP3 by immunohistochemistry on both frozen and paraffin-embedded tissues. However, this reagent may only detect a subpopulation of FOXP3+ cells.

1.4 The FOXP3 mAbs recognize the FOXP3 protein in a variety of applications and two are cross-reactive with the murine Foxp3 protein

The FOXP3 mAbs were tested for their ability to recognize formalin-resistant epitopes by immunohistochemistry using routinely fixed tonsil and formalin-fixed paraffin-embedded pellets of FOXP3-transfected COS-1 cells. All of the mAbs recognized the FOXP3 protein in routinely fixed tissues and labeled FOXP3 transfectants. Some mAbs gave less non-specific background staining than others, whereas some showed stronger labeling of positive cells, these data are summarized in Table 2. Abs 86D/D6 and 236A/E7 are recommended for immunohistochemistry on paraffin-embedded tissues.

Table 2. Reactivity of FOXP3 mAbs

Antibody	IH FOXP3 transfectants	IH FOXP1, 2 or 4 transfectants	IH tonsil frozen	IH paraffin tonsil	Western blotting transfectants	Flow cytometry transfectants	Murine tissues
86D/D6	+	–	+	++	+	+	–
150D/E4	+	–	+	+	+	+++	+
157B/F4	+	–	+	++[a)]	+	++	–
206D/B1	+	–	+	+	+	++	–
221D/D3	+	–	+	++[a)]	+	+++	+
236A/E7	+	–	+	++	+	+++[a)]	–
259D/C7	+	–	+	++[a)]	+	+++[a)]	–

[a)] Indicates that there was some nonspecific background staining. IH, immunohistochemistry.

The ability of the mAbs to recognize the FOXP3 protein by flow-cytometry and by Western blotting was also tested using FOXP3-transfected COS-1 cells. All the mAbs recognized the recombinant FOXP3 protein to some extent in Western blotting and in flow cytometry (Table 2). The human and murine FOXP3 proteins are approximately 87% identical, thus the FOXP3 mAbs were also tested for reactivity with the murine Foxp3 protein. The mAbs 150D/E4 and 221D/D3 were found to be cross-reactive with

the murine Foxp3 protein and specifically labeled CD4+ T cells in murine spleen and lymph nodes (data not shown as these are consistent with the human data).

1.5 Characterization of FOXP3 protein expression in normal human tissues

FOXP3 protein expression was assessed by immunohistochemistry with mAb 236A/E7 on a routinely fixed normal-tissue microarray containing 39 different human tissues and on whole sections of lymphoid tissues. Tissues included tonsil, spleen, bone marrow, brain, larynx, parotid gland, thyroid, gall bladder, liver, lung, skin, skeletal muscle, kidney, pancreas, stomach, colon, duodenum, small intestine, bladder, ovary, uterus, breast, placenta, prostate, testis, fetal liver and fetal thymus. There was no nuclear FOXP3 protein expression observed in the range of normal non-hematological tissues tested, with the exception of scattered positive lymphocytes in colon, stomach, and fallopian tube. These data are consistent with the reported restricted expression of FOXP3 within lymphocytes. Within hematological tissues there were much higher numbers of lymphocytes expressing FOXP3. The FOXP3+ cells were scattered within the interfollicular areas of tonsil (Figure 2A) and were occasionally seen within the follicular germinal centers (Figure 2A). Reactive lymph node contained many FOXP3+ cells and these were distributed throughout the tissue, including within the mantle zone and germinal centers of the secondary follicles. In spleen there were occasional FOXP3+ cells in the red pulp and there were increased numbers in the T cell areas around vessels (Figure 2A). There were many FOXP3+ cells in fetal thymus and in the mature thymus these were present predominantly in the medulla with only scattered FOXP3+ cells being present in the cortex (Figure 2A). In bone marrow a small number of FOXP3+ cells were also observed. To confirm the immunophenotype of the FOXP3+ cell population in situ within tissues, double-labeling studies were performed by immunofluorescent (Figure 2B) and immunoenzymatic techniques. These studies confirmed that in tonsil the FOXP3 protein was expressed exclusively in the CD3+ T cell population and no double-labeling of CD20+ B cells was observed. The majority of FOXP3+ cells were, as previously reported, both CD4+ and CD25+. However, double-labeling identified a very small population of FOXP3+CD8+ cells and indicated that a minority of FOXP3+ cells were CD25- (Figure 2B)

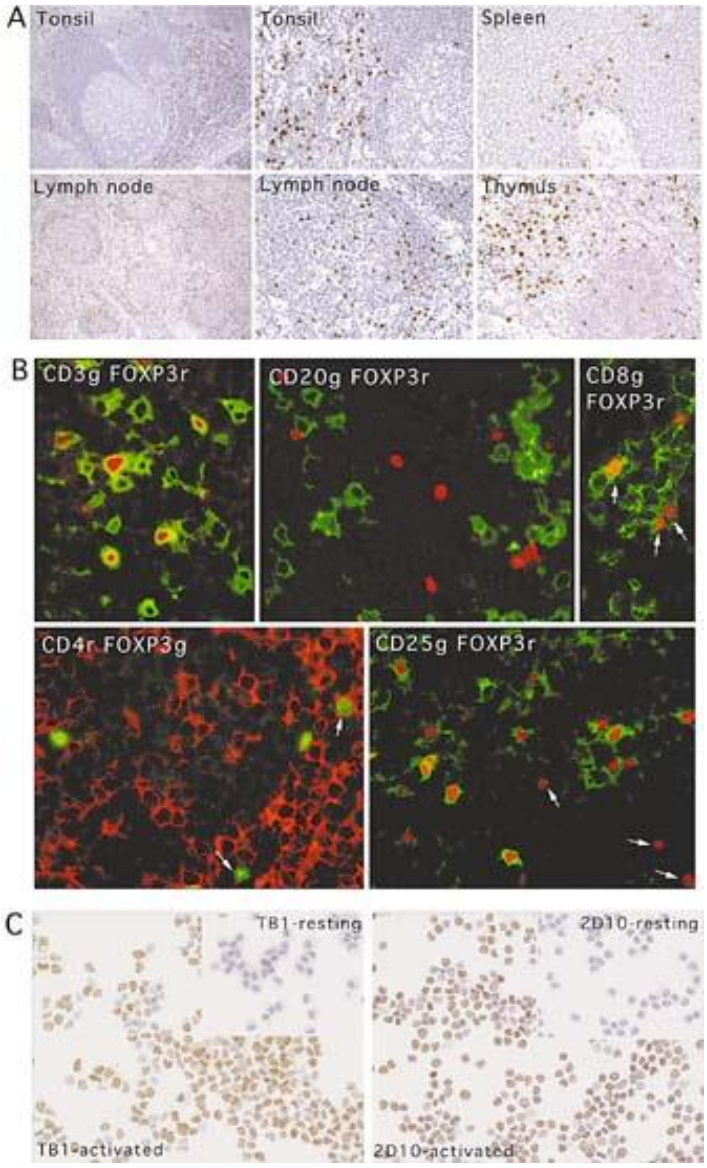


Figure 2. FOXP3 protein expression in normal human tissues. (A) Illustrates peroxidase immunolabeling of normal lymphoid tissues with the 236A/E7 FOXP3 Ab. (B) Illustrates double-immunofluorescent labeling of normal tonsil (g, green; r, red), confirming that the FOXP3 mAb recognizes predominantly CD4+CD25+ T cells. White arrows in the CD8 picture indicate the presence of rare CD8+FOXP3+ cells and in the CD25 picture these indicate that a proportion of the FOXP3+ cells are CD25–. (C) Illustrates both activated and resting cells from the CD4+ T cell clone TB1 (left) and the CD8+ clone 2D10 (right) stained for FOXP3 protein expression showing that activation induces FOXP3 expression.

1.6 FOXP3 is expressed in activated T cells

There have been reports that FOXP3 expression is induced in activated T cells. Cytospin preparations of both resting and activated CD8+ and CD4+ human T cell clones were immunostained with FOXP3 mAb 236A/E7. FOXP3 protein expression was restricted to the nuclei of activated CD4+ and CD8+ T cell clones and was absent in the resting cells (Figure 2C).

1.7 Frequency of FOXP3 expression in the human CD4+ population

CD4+CD25+ and CD4+CD25- populations were purified from peripheral blood taken from three individuals without any known disease as described in the "Materials and Methods" section. Immunofluorescent labeling of FOXP3 expression was

performedand and the numbers of FOXP3+ and FOXP3- cells were scored in each sample. Approximately half of the CD4+CD25+ population was FOXP3+ with a frequency of 55.7±5.2% (Figure 3A). In contrast, only 3.6±0.9% of the CD4+CD25- cells expressed FOXP3.

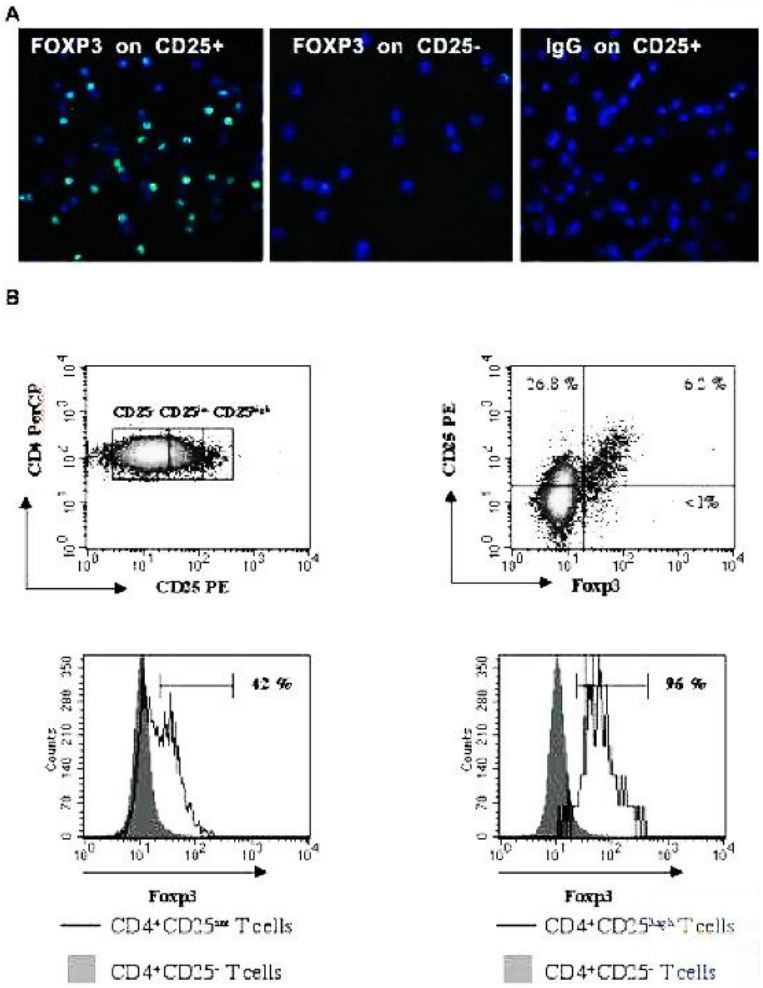


Figure 3. Frequency of FOXP3 expression in CD4+ T cells. (A) Illustrates the frequency of FOXP3+ cells within the CD4+CD25+ (left) and CD4+CD25– (middle) populations purified from peripheral blood No staining of the CD4+CD25+ cells was observed with the secondary Ab alone (right). (B) The top left panel shows the FACS plots and gating of PBL labeled with CD4 and CD25. The top right panel represents the FOXP3 expression according to CD25 expression in the whole CD4 population. The FOXP3 expression in the gated populations is illustrated in the histograms. Quadrants are drawn based on isotype-matched control antibodies that gave <1% background. Representative of three separate experiments.

Despite early difficulties with detecting endogenous FOXP3 protein by flow cytometry, a modified staining protocol that included a DNAse digestion step enabled FACS analysis of endogenous FOXP3 protein expression in the CD4+ population and correlation with CD25 expression (Figure 3B). In peripheral blood, 95.7% (94.7–95.8) of

CD4+CD25high T cells expressed FOXP3 whereas only 34.9% (22.3–47.4) of CD4+CD25int T cells stained positive for FOXP3. No detectable staining was observed in the CD25- population or in resting CD8+ T cells (data not shown). As in the pheripheral blood lymphocytes, the majority (>95%) of thymic CD4+CD25high T cells express FOXP3 (data not shown). It is claimed that the commercially available hFOXY mAb is also suitable for FACS analysis of FOXP3 expression. However, we were unable to reveal FOXP3 expression amongst peripheral blood CD4+CD25+ cells using this reagent, suggesting it is not suitable for this application (data not shown).

1.8 The FOXP3 mAb label functional CD4+CD25+T cells

Although the double-labeling studies indicate that the FOXP3 mAb label predominantly CD4+ CD25+ T cell, their ability to detect T cells with functional regulatory activity was also confirmed. The immunophenotype of CD4+CD25+ T cells purified from peripheral blood using magnetic-bead selection was confirmed by flow cytometry (Figure 4A). Nuclear expression of the FOXP3 protein was confirmed by immunofluorescent labeling (Figure 4B), only the occasional FOXP3+ cell was observed in the CD25- population and no staining was observed when using the isotype-matched MR12 control mAb (data not shown). These FOXP3+ cells were able to suppress the proliferation of CD4+CD25– T cells (Figure 4C) confirming that the mAb label functional CD4+CD25+FOXP3+ T_{reg} cells.

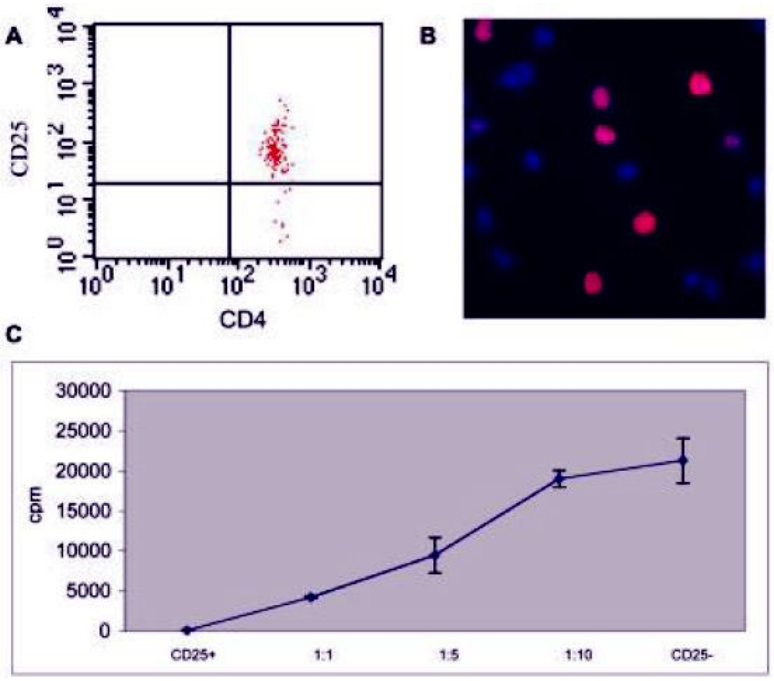


Figure 4. The FOXP3 236A/E7 mAb labels functional suppressor T cells. (A) Illustrates FACS confirmation of the CD4+CD25+ phenotype of the bead-sorted suppressor cells. (B) Shows the labeling of these CD4+CD25+ cells with the 236A/E7 FOXP3 mAb.(C)The CD4+CD25+FOXP3+ cells are functionally able to suppress the proliferation of CD4+CD25–FOXP3– cells in a dose-dependent manner

1.9 FOXP3 expression in lymphomas

Results of the examination of FOXP3 protein expression in lymphomas are shown in Table 3. In the 89 B- and 58 non-ATLL T-cell lymphomas, FOXP3 expression was only detected in the reactive T-cell component (Figure 5A), without any noticeable staining in the tumoural cells (Figure 5B–D). The proportion of FOXP3-positive reactive T-cells was highly variable, as compared with normal reactive tissue, ranging from almost a complete absence (usually seen in Burkitt's lymphoma, Figure 5D) to an evident infiltrate (common in follicular lymphoma, Figure 5C). In contrast, FOXP3 expression in the tumour cells was detected in 17/25 (68%) ATLL cases. In all these cases, FOXP3 expression was detected in CD4/CD25 positive cells (Figure 6 and Table 4)

Table 3. FOXP3 protein expression in lymphomas

Tumour type	*Number of cases total: 172*	*Proportion of cases showing FOXP3 expression by tumour cells*
T-cell lymphomas	83	
T-cell lymphoblastic lymphoma	4	0/4
Peripheral T-cell lymphoma	20	0/20
Angioimmunoblastic lymphoma	5	0/5
T/NK cell lymphoma (nasal type)	5	0/5
Anaplastic large cell lymphoma (ALK+)	4	0/4
Anaplastic large cell lymphoma (ALK–)	5	0/5
Mycosis fungoides	15	0/15
Adult T-cell leukaemia (ATLL)	25	8/25 strong expression 9/25 intermediate expression 8/25 no expression
B-cell Lymphomas	89	
B-Cell lymphocytic leukaemia	14	0/14
Marginal zone B-cell lymphoma	12	0/12
Follicular lymphoma	17	0/17
Mantle cell lymphoma	14	0/14
Diffuse large B-cell lymphoma	17	0/17
Burkitt's lymphoma	15	0/15

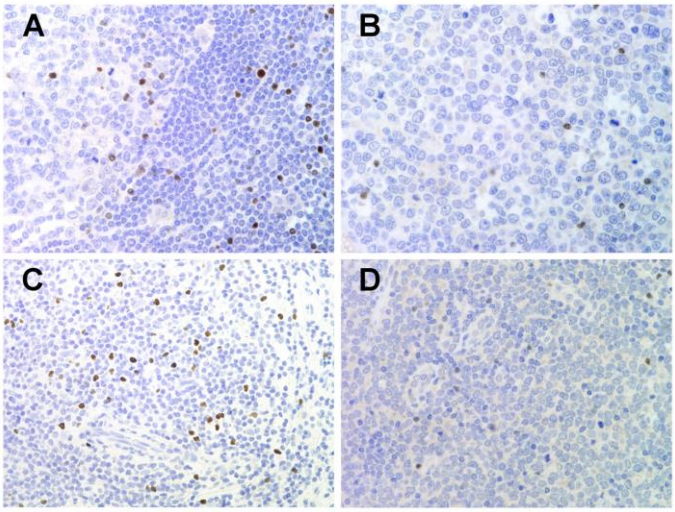


Figure 5: FOXP3 expression in normal and tumoural tissues.
(A) Strong nuclear FOXP3 expression in reactive tonsil: most positive cells arelocated in the interfollicular T-cell area. (B) Low presence of reactive FOXP3þ T-cells in a case of peripheral T-cell lymphoma; tumoural cells are negative. (C) Follicular lymphoma: a significant number of reactive FOXP3+ T cells are detected in neoplastic follicles; tumoural cells are negative. (D) Low presence of reactive FOXP3+ T cells in Burkitt's lymphoma; tumoural cells are negative.

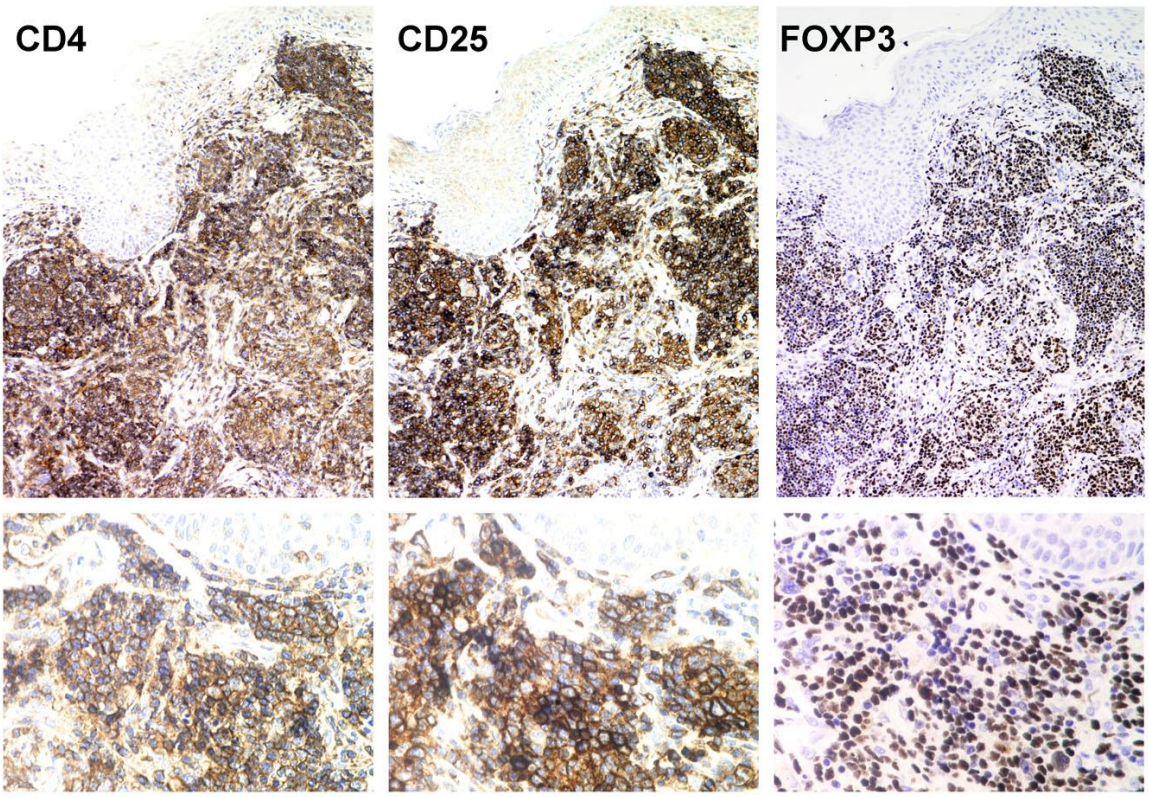


Figure 6. FOXP3 expressions in ATLL**.** Skin infiltration by tumour cells (case number 10) showing a large proportion of CD4+, CD25+ and FOXP3+ cells (immunohistochemistry in paraffin-embedded tissue).

Table 4. Study of FOXP3 expression in ATLL.

Case number	*Site*	*HTLV-1 Southern*	*ATLA*	*Main phenotype*	*FOXP3 immunostaining*	*Follow up (months)*
1	LN	+		CD4+, CD25+, CD7-, CD8-, CD30+	Intermediate positivity	DOD (25)
2	LN	+		CD4+, CD7-, CD8- CD25NA	Intermediate positivity	DOD (12)
3	LN	+		CD4+, CD25+, CD7-, CD8-	Intermediate positivity	DOD (2)
4	LN	?	+	CD4-, CD7-, CD8-, CD3+ CD25NA	Intermediate positivity	DOD (16)
5	LN	+		CD4+, CD25+, CD7-, CD8-, CD30+	Intermediate positivity	DOD (6)
6	LN	+		CD4+, CD25+, CD8+, CD3+	Intermediate positivity	DOD (4)
7	LN	+		CD4+, CD25+, CD7-, CD8-	Intermediate positivity	DOD (36)
8	LN	+		CD4+, CD7-, CD8- CD25NA	Intermediate positivity	DOD (65)
9	LN	+		CD4+, CD25+, CD7-, CD8-	Intermediate positivity	DOD (6)
10	Skin	+		CD4+, CD25+, CD7-, CD8-	Strong positivity	AWD (30)
11	Skin	?	+	CD4+, CD25+, CD7-, CD8-, CD30+	Strong positivity	DOD (3)
12	LN	+		CD4+, CD7-, CD8- CD25NA	Strong positivity	DOD (6)
13	LN	+		CD4+, CD25+, CD7-, CD8-	Strong positivity	DOD (6)
14	LN	+		CD4+, CD25+, CD7-, CD8-	Strong positivity	DOD (8)
15	LN	+		CD4+, CD25+, CD7-, CD8-	Strong positivity	DOD (51)
16	LN	+		CD4+, CD25+, CD7-, CD8-	Strong positivity	DOD (5)
17	Subcutis	+		CD4+, CD25+, CD7-, CD8-	Strong positivity	DOD (38)
18	LN	+	+	CD4+, CD7-, CD8- CD25NA	Negative	DOD (133)
19	LN	+		CD4+, CD25-, CD7-, CD8-	Negative	DOD (49)
20	LN	+		CD4+, CD7-, CD8- CD25NA	Negative	DOD (54)
21	LN	+		CD4+, CD25+, CD7-, CD8-	Negative	DOD (45)
22	LN	?	+	CD25+CD4+	Negative	DOD (15)
23	LN	+		CD4+, CD25+, CD7-, CD8-	Negative	DOD (37)
24	LN	+		CD4+, CD25-, CD7-, CD8-	Negative	DOD (6)
25	LN	+		CD4+, CD25+, CD7-, CD8-	Negative	DOD (1)

DOD: death of disease; AWD: alive with disease; LN: Lymph node, NA: not available.

ATLA: ELISA detection of anti-HTLV-1 antibodies.

In terms of FOXP3 expression, three subgroups of ATLL cases could be defined taking into account both the proportion of positive cells and the intensity of the expression. The first subgroup (32% of patients) represents samples with strong nuclear FOXP3 expression in the majority of tumour cells (Figure 7A and B). The second group (36% of patients) exhibited intermediate expression of FOXP3 tumoural cells (Figure 7C and D), with a variable intensity of expression. Expression was weaker than observed in the first group and lower than observed for the scattered FOXP3+ cells present in the reactive component. Although in the majority of these cases, the distinction between tumoural and reactive FOXP3+ T-cells is straightforward, some samples needed detailed observation for evaluation. The presence of FOXP3 in tumoural cells was confirmed by double fluorescence analyses, showing that FOXP3+ nuclei are found in a minority of atypical CD25+ cells (Figure 7E). Finally, the third group of cases (32% of patients)

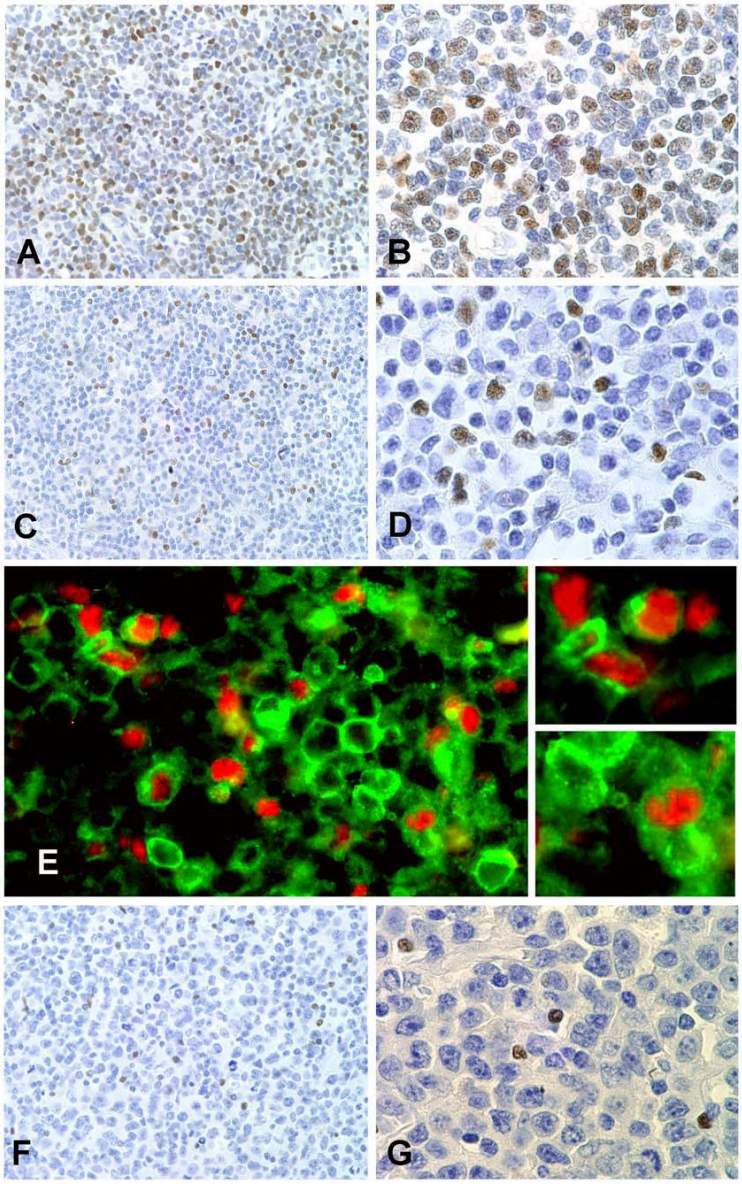


Figure 7. FOXP3 expression in Adult T-cell leukaemia (ATLL) cases (A and B) Strong FOXP3 expression can be observed in the nucleus of the tumour cells. (C and D) Intermediate FOXP3 expression can be observed in some tumour cells. (E) Indirect double immunofluorescence in the same case displayed in panels c and d show nuclear FOXP3 expression (red) in a minority of large atypical CD25 positive cells (green). (F and G) No FOXP3 expression is observed in the tumour cells of this ATLL case.

comprised ATLL samples where FOXP3 expression was clearly absent in the tumour cells (Figure 7F and G).

1.10 Survival analysis

As has been commonly reported, the course of ATLL is generally rapidly progressive and ultimately fatal with only one of the patients remaining alive at the follow-up (OS ranging from 1 to 133 months). The analysis of the relationship between FOXP3 protein expression and survival only showed a near significant association with survival: patients with FOXP3-negative tumours tending to have better survival (median OS 41 months (range 1–133) vs 7 months (range 2–65); P=0.060) than those with strong or intermediate FOXP3 expression (Figure 8). There was no significant difference in OS between the groups with intermediate or strong FOXP3 expression.

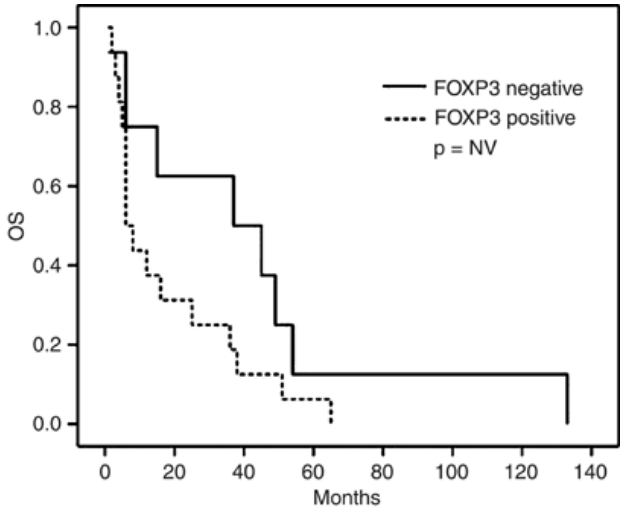


Figure 8. Kaplan–Meier analysis of overall survival (OS) in ATLL patients grouped according to the expression of FOXP3: shorter survival in the FOXP3-positive group than in the FOXP3-negative group.

2. Results second project: Follicular helper T cells

2.1 Immunostaining of SAP and NAT in normal lymphoid tissue

The expression of the markers PD-1 and SAP in human lymphoid tissue (studied by single and double labeling techniques) is illustrated in Figure 9 and 10 and summarized in Table 5. Staining was performed on paraffin-embedded tissue sections. We confirmed that PD-1 was preferentially expressed by scattered cells in germinal centers,

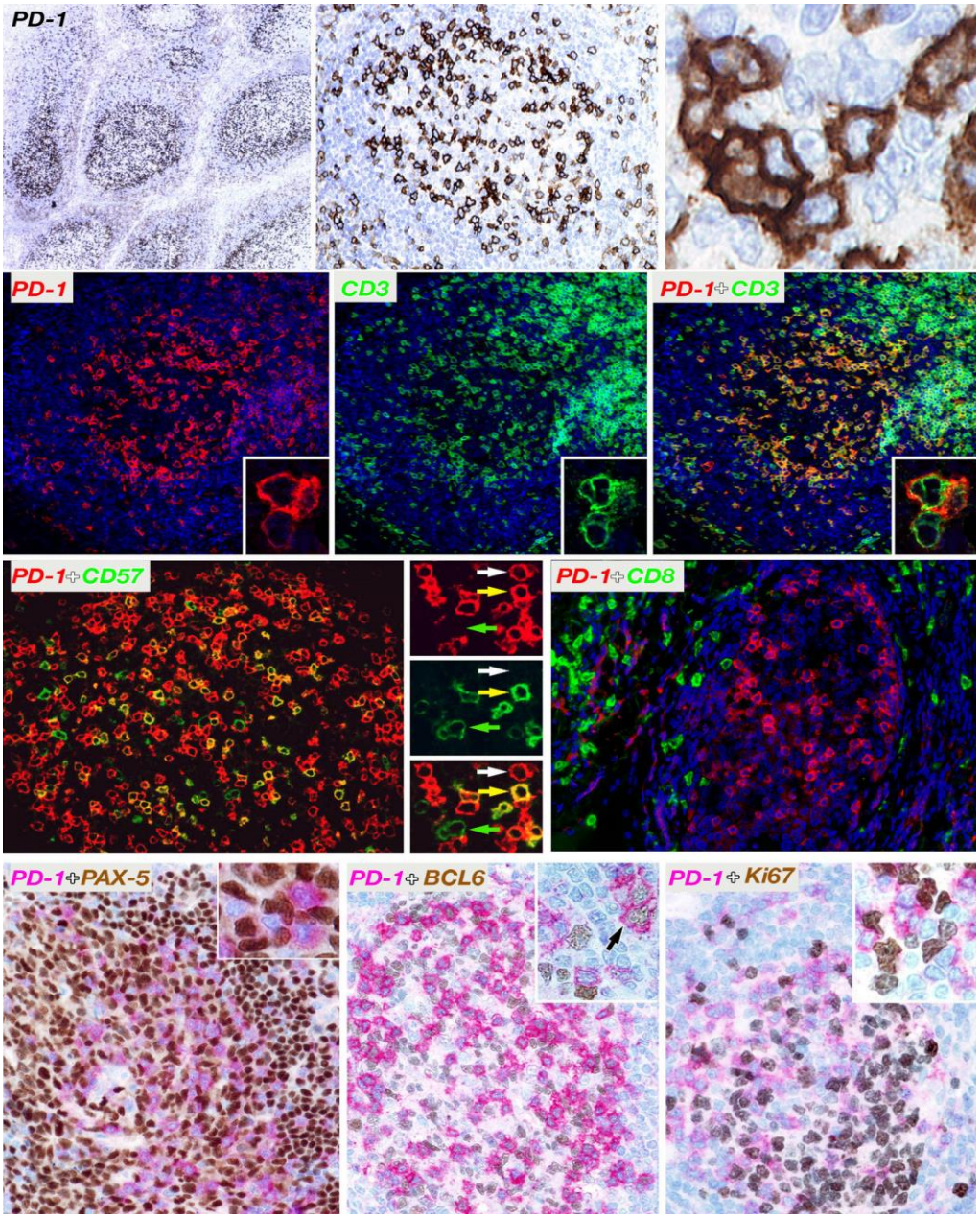


Figure 9. Expression of PD-1 by lymphoid cells.
First row: Immunoperoxidase staining of human tonsil (seen at low, intermediateand high power) shows strong labeling of many cells within germinal centers. Second row: Double immunofluorescent staining of the PD-1-positive cells (red) in the germinal center co-express CD3 (green. Third row (left): Double labeling for PD-1 (red) and CD57 (green) in a germinal center (left). Double labeling for PD-1 (red) and CD8 (green) shows reciprocal expression of the two molecules in intrafollicular T cells (right). Fourth row: Double immunoenzymatic staining shows (left) the reciprocal expression of PD-1 (red) and PAX-5 (nuclear, brown) in B cells, and also that a proportion of PD-1-positive germinal center T cells (red) express nuclear BCL6 (brown - example arrowed), and (right) that they lack the proliferation marker Ki-67.

and these cells were shown by double labeling for CD3 to be T cells. Outside lymphoid follicles, the great majority of T cells were PD-1-negative (or only weakly positive).

Additional double immunostaining showed that many PD-1-positive follicular T cells lacked CD57, CD8, the B-cell transcription factor PAX-5 and the proliferation marker Ki67 (Figure 9). However, approximately 20% of the PD1-positive follicular T cells expressed BCL6 (Figure 10) (although the proportion of BCL6- positive cells varied between germinal centers). The pattern of staining observed for the adaptor molecule SAP (Figure 10) was very similar to the pattern observed for PD-1, and double staining for the two markers showed essentially identical labeling within germinal centers. In extrafollicular regions SAP was expressed by occasional T cells that were PD-1 negative (Figure 10).

Table 5. PD-1 and SAP in normal lymphoid tissue.

Marker	*Tissue staining distribution*			*Subcellular localization pattern*
	Tonsil	*Thymus*	*Spleen*	
PD-1	Germinal center T cells. Scattered extrafollicular cells, usually more weakly stained.	Scattered cells in the medulla.	T cells in lymphoid areas. Rare cells in the red pulp.	Membrane-associated.
SAP	Germinal center T cells. Scattered extrafollicular cells, usually more weakly stained.	Majority of cortical thymocytes. Scattered cells in the medulla.	T cells in lymphoid areas. Scattered cells in the red pulp.	Cytoplasmic, sometimes nuclear.

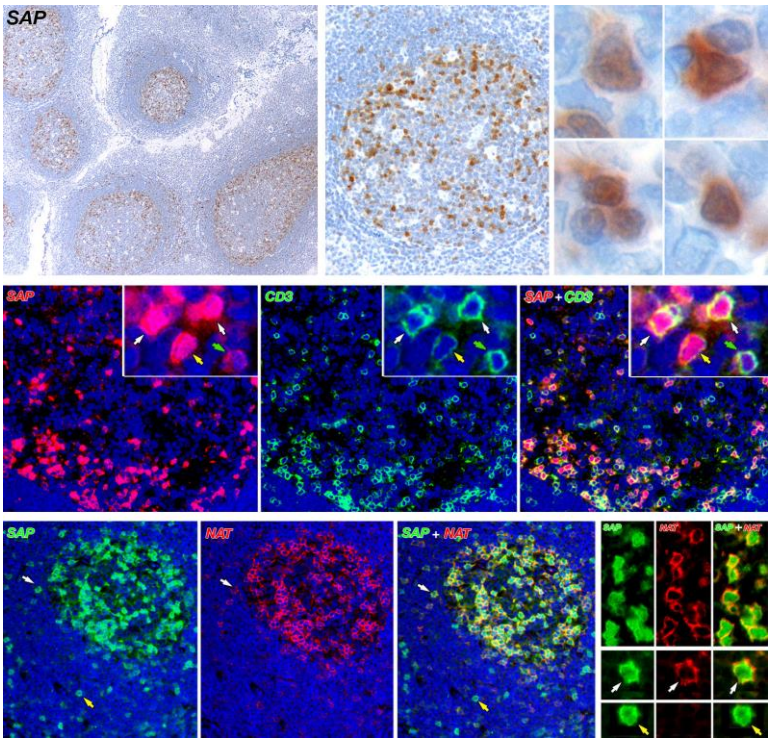


Figure 10. SAP expression in normal human tonsil.
Upper row: Immunperoxidase staining (viewed at low, intermediate and high magnification) shows a population of strongly stained cells within germinal centers with cytoplasmic and nuclear labeling. Second row: Double immunofluorescent staining for SAP (red) and CD3 (green) shows that many cells co-express these markers (white arrows). Cells expressing SAP with little or no CD3 are also present (yellow arrows) together with SAP-weak/negative CD3-positive cells (green arrows). Third row: Double immunofluorescence labeling for SAP (green) and PD-1 (red) shows that many cells in the germinal center co-express both molecules.

2.2 Western Blotting

Western blotting of cell lysates from the YT line with the anti-PD-1 antibody NAT showed a single band with a molecular weight of approximately 47 kDa (Figure 11. Anti-SAP was tested against the YT line and also against a number of T-cell lymphoblastic cell lines (Jurkat, CCRF/CEM and Molt-4). A band of approximately 15 kDa was found in each of these, accompanied in the case of Jurkat and CCRF/CEM with a second band of slightly smaller size (Figure 11). In contrast, the ALK-positive lymphoma cell line Karpas 299 was SAP-negative.

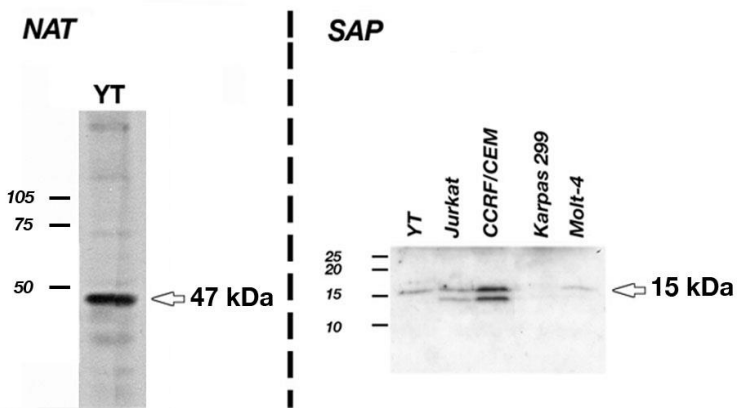


Figure 11. Western blotting analysis of antibody NAT and SAP.
Blotting with the former antibody in the NK cell-derived cell line YT shows a band with a molecular weight of approximately 47 kDa. SAP is detectable as a 15 kDa band in the YT line and also in three T-cell lymphoblastic cell lines (Jurkat, CCRF/CEM and Molt-4). Karpas 299 (an ALK-positive lymphoma cell line) is negative. A second band, slightly smaller than the 15 kDa band, is also visible in the Jurkat and CCRF/CEM cell lines.

2.3 Immunostaining of PD-1 and SAP in neoplastic lymphoid tissue

Among T-cell neoplasms, PD-1 and SAP were expressed over three quarters of the cases of AITL tested (42/49 and 59/69 cases, respectively) (Table 6 and Figure 12). The two markers were expressed independently of each other, with the result that almost 95% of cases (40/42) expressed at least one of these markers (Table 6 and Figure 12). In addition to AITL both PD-1 and SAP were found in a minority of cases of peripheral T-cell lymphoma (5/30 and 13/37, respectively) (Table6 and Figure 11). Twenty-two cases that had been investigated for both markers were reviewed, and two cases, each of which was positive for both PD-1 and SAP (Table 6), showed some features

consistent with atypical AITL, but a further six cases (two double positive and four positive for SAP alone) appeared to be classic examples of peripheral T-cell lymphoma. Among the samples tested for only marker, a further six cases expressing PD-1 or SAP were identified, and two of these (one PD-1 positive, one SAP-positive) also had some features on review suggestive of possible AITL. All cases of AITL were immunostained for the CXCL13 chemokine, and many cases contained CXCL13-positive neoplastic cells. However, background extracellular staining was often present and labeling in many positive cells was restricted to a single cytoplasmic dot. In consequence, evaluation of immunostaining in the majority of cases was more difficult than for and SAP. (Table 6 and Figure 11). SAP was expressed in 15/21 cases of T lymphoblastic lymphoma . (Table 6 and Figure 11) (whereas PD-1 was not found in this tumor type). In contrast, PD-1 was found in 5/9 cases of mycosis fungoides, whereas SAP expression was limited to a small minority of the neoplastic cells in 3/6 cases. (Table 6 and Figure 11). SAP and PD-1 were absent from

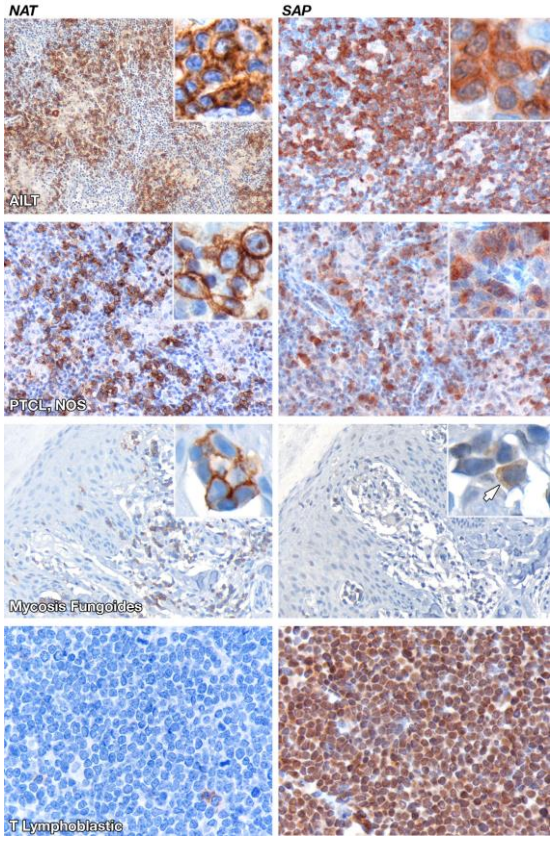


Figure 11 .Immunostaining of T cell lymphomas for PD-1 and SAP. *Top row*: Two angioimmunoblastic T-cell lymphomas (AITL) were positive for both markers. *Second row*: a case of peripheral T cell lymphoma expresses PD-1 and SAP. *Third row*: neoplastic cells in a case of mycosis fungoides express PD-1 (inset, high magnification) but are negative for SAP, with the exception of a few weakly positive cells (arrowed) (inset, high magnification). *Fourth row*: a case of T lymphoblastic lymphoma shows extensive nuclear staining for SAP.

Table 6. PD-1 and SAP in Lymphomas

	PD-1	*SAP*
Lymphoma/leukemia type		
T/NK cell non-Hodgkin's		
Lymphoblastic (T)	0/20	15/21
Peripheral	5/30[a]	13[d]/37
Intestinal	1/10	3[e]/10
Angioimmunoblastic T cell (AITL)	42/49	59/69
Natural killer (NK)	1[b]/8	3/5
Mycosis fungoides	5/9	0/6[f]
ALK-positive	0/4	1[g]/13
ALK-negative, ALCL	0/1	1[h]/7
B cell non-Hodgkin's		
Lymphoblastic (B)	0/10	0/11
Chronic lymphocytic (CLL)	0/13[c]	0/20
Mantle cell	0/14	0/20
Follicular (Grade 1, 2, 3)	0/70	0/114
Burkitt's	0/21	0/14
Diffuse large	3/98	3/115
Marginal zone (nodal and splenic)	0/14	0/23
MALT	0/8	0/12
Hairy cell	0/1	0/1
Myeloma/plasmacytoma	0/2	0/10
Hodgkin's		
Classical	0/18	1/21
Lymphocyte predominant	0/11	4[i]/14

Table 7. Comparison between PD-1 and SAP

Lymphoma/ leukemia type	*PD-1-pos/ SAP-pos*	*PD-1-pos/ SAP-neg*	*PD-1-neg/ SAP-pos*	*PD-1-neg/ SAP-neg*
T lymphoblastic	–	–	9/13	4/13
Peripheral T-cell lymphoma	4/22	–	4/22	14/22
Intestinal	-	1/10	3/10	6/10
Angioimmunoblastic T cell lymphoma	31/42	4/42	5/42	2/42
Natural killer (NK)	–	1^/3	2/3	–
Mycosis fungoides	–	4/5	–	1/5
ALK-positive	–	–	1*/3	2/3
ALK-negative	–	–	1$/1	–

*^Approximately 50% of the tumor cells were positive; * The tumor cells were weakly positive; $ The tumor cells were very weakly positive.*

almost all of the non-Hodgkin's B cell neoplasms investigated (Table 6).

PD-1 was found on the tumor cells in three diffuse large B-cell lymphomas (out of 98) and was also present in a minority of larger cells in cases of chronic lymphocytic leukemia/lymphoma (CLL). In six out of the 13 CLL cases, proliferation centers were recognizable and it was clear that the large PD-1 positive cells belonged to these structures. SAP was absent from all cases of non-Hodgkin's B cell lymphoma, with the exception of three cases (out of 115) of diffuse large B-cell lymphoma (one of these three cases was PD-1 negative, the other two were not evaluated for PD-1) and proliferation centers in CLL (although the staining was weaker than for PD-1 and the number of SAP-positive cells was lower). Moreover, weak labeling of neoplastic cells for SAP was observed in four out of 14 cases of lymphocyte predominant Hodgkin's disease (in approximately just over half of the tumor cells). Reed-Sternberg cells were SAP-negative with the exception of one case (out of 21) of classical Hodgkin's disease in which approximately 50% of the tumor cells were weakly to moderately positive.

2.4 Infiltrating cells

In all studied B-cell lymphomas, occasional non-neoplastic cells carrying PD-1 and SAP were observed. The highest percentage of PD-1 negative and SAP positive infiltrating cells was found in follicular lymphomas, lymphocyte predominant Hodgkin's disease (Figure 12) and in some cases of diffuse large B-cell lymphoma. In the first disease, the number of these infiltrating cells varied widely from case to case and was independent of histologic grade. In lymphocyte predominant Hodgkin's disease, PD-1 and SAP were positive in infiltrating cells, and in many cases these cells formed rosettes surrounding the neoplastic cells (Figure 12). Incontrast, in classical Hodgkin's disease, PD-1 positive or SAP positive rosettes were found in only a minority of cases (Figure 12).

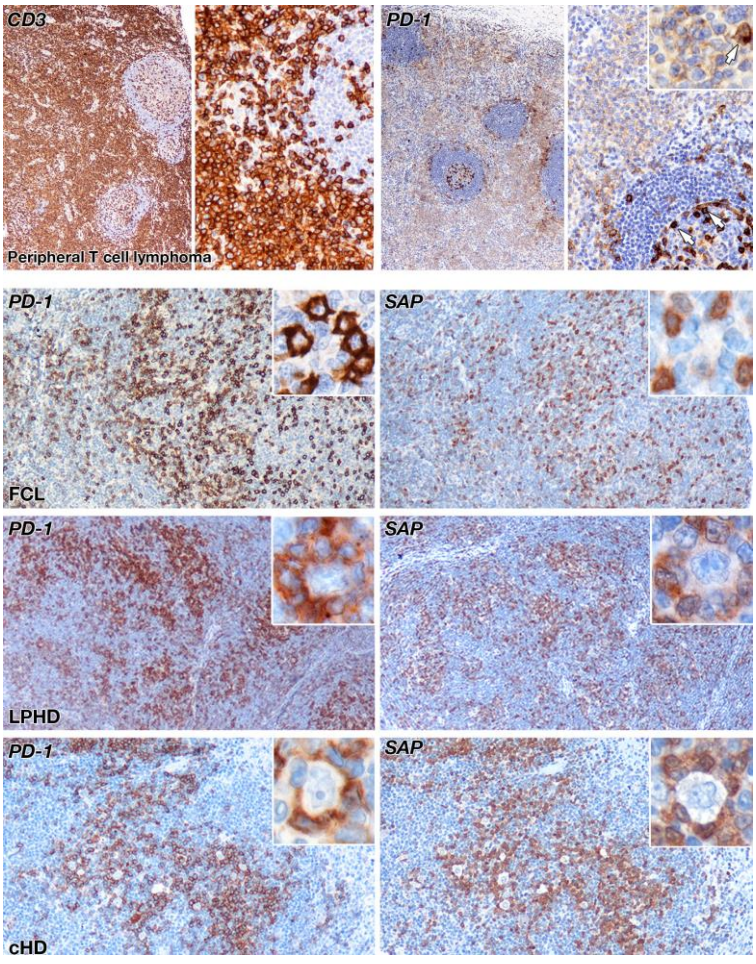


Figure 12. Immunostaining of T cell lymphomas for PD-1 and SAP.
Top row, left: The positive CD3 staining of tumor cells in a case diagnosed as typical peripheral T-cell lymphoma highlights its interfollicular growth pattern and the preservation of lymphoid follicles. *Top row, right*: the tumor cells in the same case show weak to moderate expression of PD-1. Note the difference in terms of intensity between the strongly positive normal T cells (arrowed) in a germinal center and the weaker staining of tumor cells (as also illustrated at high magnification in the inset) adjacent to the unstained mantle zone cells. *Second row*: in a case of follicular lymphoma, many PD-1- and SAP-positive cells are seen in a neoplastic follicle. *Third row*: in a case of lymphocyte predominant Hodgkin's disease (LPHD) many reactive lymphocytes are PD-1 negative and SAP-positive. The insets show cells expressing these markers forming rosettes surrounding the tumor cells. *Third row*: in a case of classical Hodgkin's disease (cHD) PD-1 and SAP are seen in many reactive lymphocytes, and the insets show neoplastic cells surrounded by rosettes of PD-1 negative and SAP-positive T cells.

2.5 PD-1, a Follicular T-cell Marker useful for recognizing nodular lymphocyte-predominant Hodgkin lymphoma

2.5.1. PD-1 in the differential diagnosis of B cell Lymphomas

The potential diagnostic value of PD-1 was explored in a series of 152 cases diagnosed as nodular sclerosis Hodgkin lymphoma, mixed cellularity Hodgkin lymphoma, lymphocyte rich classic Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL), and T-cell/histiocyte rich B-cell lymphoma (T/HRBCL). The results obtained are summarized in Table 8.

Table 8. Main immunohistochemical features

Lymphoma Type	No. Cases	CD57 Rosettes	PD-1 Rosettes	CXCL-13 Rosettes
NSHL	43	0*	0	0†
MCHL	14	0	0	0
LRCHL	13	8†	10	0
NLPHL	58	44	57	7‡
NLPHL with diffuse areas	7	Nodular = 7	Nodular = 5	0
		Diffuse = 0	Diffuse = 0	0
NLPHL vs. T/HRBCL	5	3†	4	0
T/HRBCL	12	0	0	0§

*3 cases positive tumoral cells.
†1 case positive tumoral cells.
‡7 cases positive tumoral cells.
§2 cases positive tumoral cells.

2.5.2 PD-1 expression in progressively transformed germinal centers (PTGCs)

The expression of PD-1 (NAT-105) was studied in paraffin-embedded tissue sections of 4 lymph nodes and 2 human tonsils biopsies. PD-1 (NAT-105) was selectively expressed by T cells in germinal centers, localized predominantly in the periphery of the germinal centers as a rim in the outer zone. Outside of lymphoid follicles, the great majority of the T cells were negative (Figure 13 A–C). PD-1 (NAT-105) was expressed by T cells in germinal centers, which were more numerous than normal or reactive germinal centers. In one case (1/7), these cells were distributed around B cells of centroblastic appearance, sometimes clearly in the form of T-cell rosettes (Figure 13 D–F).

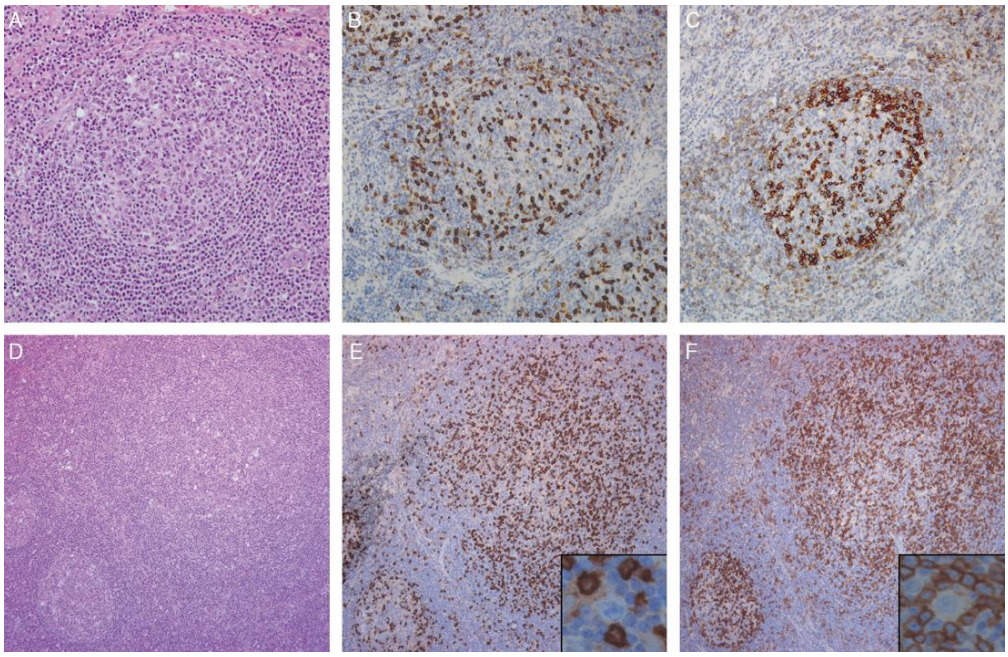


Figure 13. Reactive lymphoid follicle (A) with germinal centers stained with CD57 (B) and PD-1 (NAT-105) (C). PTGC (D) with germinal centers with frequent T cells stained with CD57 (E) and PD-1 (NAT-105), depicting isolated T-cell rosettes (F).

2.5.3 PD-1 expression in lymphocyte rich classic Hodgkin lymphoma (LRCHL)

A relatively large group of cases diagnosed as LRCHLs displayed follicular T-cell rosettes surrounding tumoral classic Reed-Sternberg cells. With CD57, 53.8% of the cases (7/13) presented follicular T-cell rosettes (Figure 14 A-B), whereas these were present in 76.9% of PD-1 cases (10/13) (Figure 14C). All cases with CD57-positive T-cell rosettes were also PD-1 positive.

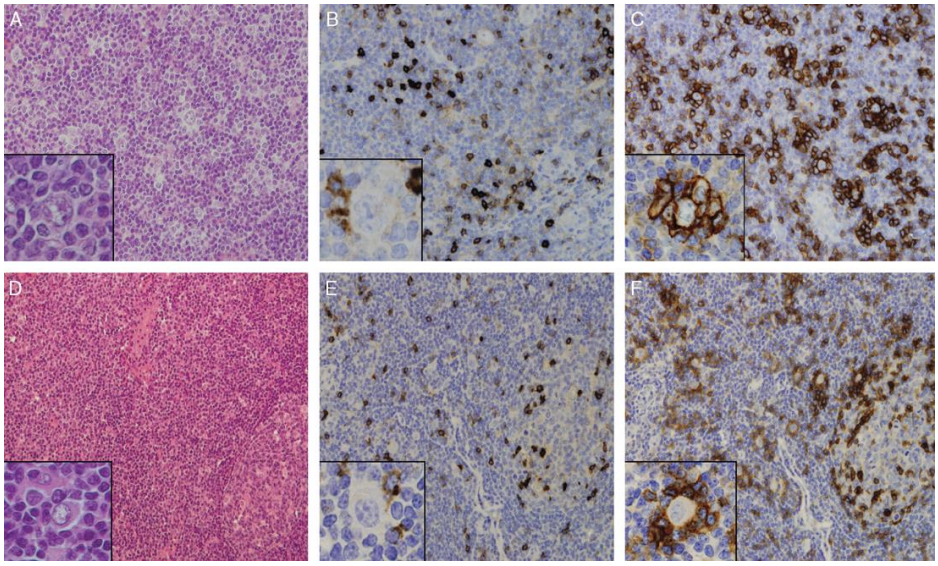


Figure 14. LRCHL (A) with CD57-negative (B) and PD-1 (NAT-105)-positive T-cell rosettes (C). NLPHL (D) with CD57-negative (E) and PD-1 (NAT-105)-positive T-cell rosettes (F).

To explain the differences between the cases with and without rosettes, clinical and immunophenotype data (Tables 9 and 10) were sought wherever possible. Clinical data obtained from 9 of 10 cases demonstrated that, independent of the presence of T-cell rosettes, most of the cases were diagnosed at a localized stage (stages I or II) and without B symptoms. Two of three cases displaying T-cell rosettes also showed B symptoms but none of these cases recurred or needed salvage therapy. No T-cell rosettes were observed with BCL6.

Table 9. Summary of the LRCHL clinical data

	LRCHL With PD-1 (NAT-105) Positive Rosettes (n = 9)	LRCHL Without PD-1 (NAT-105) Rosettes (n = 3)
Male/female	8/2	3/3
Mean age (y)	49.1	54.3
Stage I-II	7/9	2/3
Stage III-IV	2/9	1/3
B symptoms	2/9	2/3
Bulky disease	0/9	0/3
Spleen involvement	0/9	0/3
Bone marrow involvement	0/9	0/3
First-line therapy	9/9	3/3
Salvage therapy	4/9	0/2
Complete remission	8/9	3/3
Died of disease	0/9	0/3

Table 10. Main Phenotypic features of LRCHL

	LRCHL With PD-1 (NAT-105) Positive Rosettes (n = 10)	LRCHL Without PD-1 (NAT-105) Rosettes (n = 3)
CD30	10/10	3/3
CD15	5/10 (2 NE)	1/3
CD20	4/10 (2 NE)	1/3
EBV	4/10	0/3
EMA	4/10 (3 NE)	0/3 (1 NE)

EBV indicates Epstein-Barr virus; EMA, anti-epithelial membrane antigen; NE, not evaluable.

2.5.4 PD-1 expression in nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL)

In 75.9% (44/58) of the cases of NLPHL, CD57 distinct rosettes surrounding tumoral cells were seen (Figure 14D-E). With PD-1 (NAT-105), the rosettes could be demonstrated in more cases than CD57, appearing in 98.3% (57/58) of the cases (Figure 7F). Moreover, 7 cases showed CXCL-13–positive T-cell rosettes. BCL6 was positive in the tumoral cells in 6/58 cases. No BCL6–positive T-cell rosettes were observed.

2.5.5 PD-1 expression in nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) with diffuse areas

With regard to the cases of NLPHL with diffuse areas, CD57 demonstrated T-cell rosettes in all the cases (7/7), but limited to the nodular areas (Figure 15 A-B). PD-1

was positive in fewer cases than was CD57. It was also limited to the nodular areas and was only positive in 71.4% of the cases (5/7). In these cases, the intensity of the immunostaining with PD-1 seemed to diminish gradually from the nodular to the diffuse areas (Figure 15C). BCL6 was positive in the tumoral cells in most of the cases (6/7), but not in the T-cell rosettes.

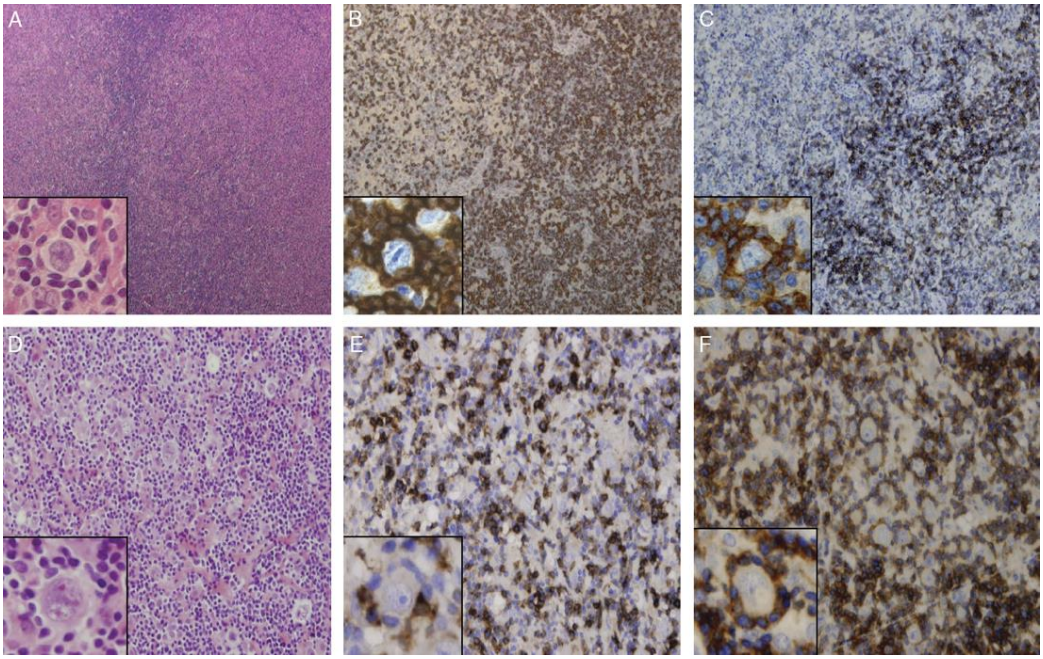


Figure 15. NLPHL with diffuse areas (A) with CD57 (B) and PD-1 (NAT-105)-positive T-cell rosettes in the nodular areas (C). NLPHL versus T/HRBCL (D) with CD57-negative (E) and PD-1 (NAT-105)-positive T-cell rosettes (F).

2.5.6 NLPHL versus T/HRBCL

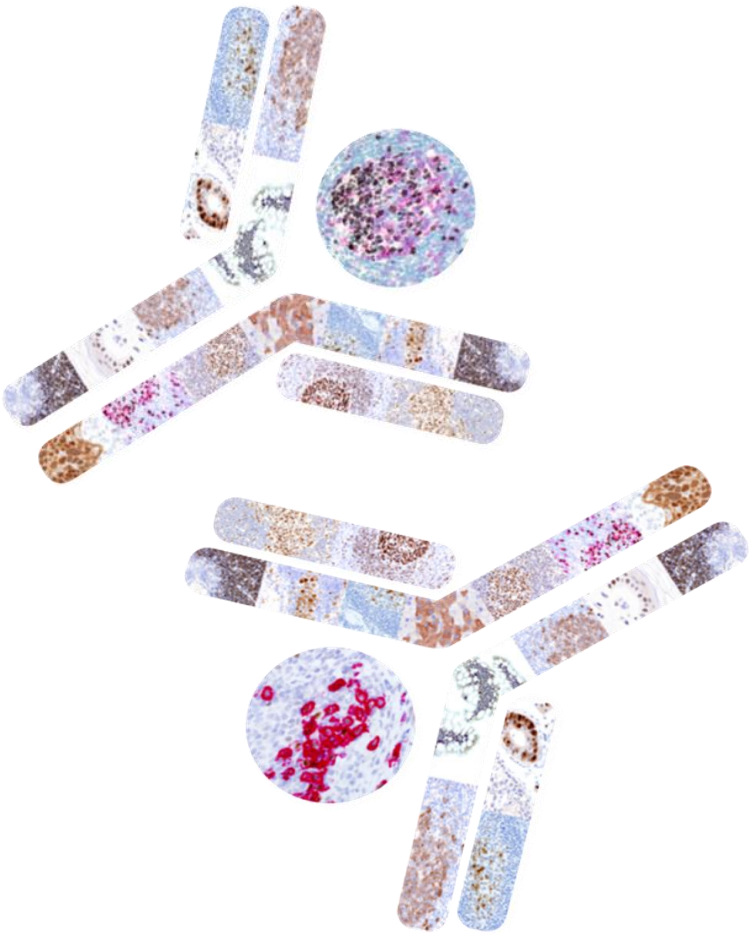
The cases not clearly defined either as NLPHL or T/HRBCL, 80% (4/5) of them displayed PD-1-positive T-cell rosettes (Figure 15D and E). As in most of the other types of lymphomas, CD57 detected fewer cases with follicular T-cell rosettes (60%; 3/5) (Figure 15E). BCL6 was positive in the tumoral cells in all cases.

2.5.7 Other types of lymphoma

Other types of lymphoma were included in this study for comparison, including NSHL, MCHL and T/HRBCL; none of them displayed T-cell rosettes either with CD57 or with PD-1.

2.5.8 Other observations

Three cases of NSHL, 1 case of LRCHL and 1 case of NLPHL versus T/HRBCL without T-cell rosettes positively immunostained for CD57 in the tumoral cells. This aberrant expression of follicular T-cell markers in the tumoral cells was also detected using CXCL-13. Thus, we diagnosed 1 case of NSHL, 7 cases of NLPHL and 2 cases of T/HRBCL with CXCL-13-positive tumoral cells. No relevant features were seen with CD10. Statistical analysis demonstrated that PD-1 was able to detect significantly more cases with T-cell rosettes, both in NLPHL and LRCHL groups, than CD57 and CXCL- 13 ($P<0.05$). The presence of PD-1–positive T-cell rosettes was significantly higher in NLPHL group than in LRCHL group ($P<0.05$). However, there was no difference with CD57 ($P>0.05$).



DISCUSSION

1. Discussion fist project

1.1 FOXP3 as a human T_{reg} marker

CD25+CD4+ T_{regs} represent a unique population of lymphocytes capable of powerfully suppressing immune responses. In healthy individuals, the most important role of T_{reg} cells is to maintain immune tolerance to self-antigens, which prevents development of autoimmune disease. It is now recognized that T_{regs} have a crucial role also in transplantation, allergic responses, microbial and cancer immunity (41, 72-74).

Since the description of suppressor T cells (now called T_{reg}) in the early 1970s, considerable progress has been made in the characterization of this cell type. The Identification, in 2003, of FOXP3 as a master regulator of T_{regs} development was fundamental for a more precise definition and characterisation of this cell population (75). At the time, due to the lack of T_{reg}-specific markers, little was known about the presence or distribution of T_{regs} in normal and tumoral human tissue.

To address these issues, we generated a panel of 7 monoclonal antibodies that specifically recognise the human FOXP3 protein. These mAbs were tested in a variety of commonly used immunological techniques, and we showed that they can be used for immunohistochemistry on frozen and paraffin-embedded tissues, western blotting and flow cytometry. Moreover, two of these mAbs can also detect the murine FOXP3 protein. The use of these antibodies has allowed us to characterise in detail the human FOXP3 expression at the single cell level in both lymphoid tissue and peripheral blood for the first time.

Immunolabelling studies on reactive human tissues showed that FOXP3 expression was restricted within the lymphoid population. In tonsil and lymph node, strong nuclear FOXP3 expression was detected in approximately 5% of interfollicular T cells, even though FOXP3+ T cells were occasionally seen within the germinal centre.

Within lymphocytes, double labelling studies have confirmed that the majority of FOXP3+ cells were indeed CD4+ CD25+ cells, and that approximately half of the whole CD4+CD25+ population in peripheral blood expressed FOXP3. A small population of CD4+CD25-FOXP3+ lymphocytes was also identified. To investigate the correlation between this FOXP3+ cell population and CD25 expression, we designed a modified staining protocol (that includes a DNAse digestion step) that enables endogenous

FOXP3 protein detection by flow cytometry. We found that 96% of CD4+ $CD25^{high}$ peripheral blood lymphocytes (PBL) were FOXP3 positive, whereas only 35% of $CD4+CD25^{low}$ PBL expressed FOXP3. These data confirmed that, in humans, T_{reg} activity is mostly restricted to the $CD4+CD25^{high}$ T cell population, and that $CD4+CD25^{low}$ are a more heterogeneous population that include also activated CD25+ lymphocytes. We also described the presence of FOXP3 protein in a small number of CD4+CD25- FOXP3+ lymphocytes, as well as in a few CD8+ T cells. Subsequent studies have confirmed the presence in humans of CD8+CD25+FOXP3+ cells with regulatory activity and able to suppress immune response (76). However, a more complete characterisation of this cell subtype remains to be done to clarify the function and rule of this cell subtype in the immune system.

Next, we were interested in determine whether the CD4+CD25+ FOXP3+ cells population selected using our FOXP3 mAbs were able to suppress CD4+CD25- cells. The results obtained confirmed that FOXP3 antibodies can be used to label functional CD4+CD25+FOXP3+ regulatory T cells.

The application of our FOXP3 mAbs for FACS analysis has opened new avenues in the investigation of the function and the differentiation of FOXP3+ T_{reg}. Although expression of the transcription factor FOXP3 currently constitutes the best-known marker for T_{regs}, a transient expression of this molecule is also observed in activated non-T_{reg} cells. The search for a specific cell surface marker for FOXP3+ cells is still an active and important area of interest.

1.2 Evaluation of FOXP3 expression in tumour cells

Nuclear FOXP3 protein expression in tumour cells was first reported in 2004 by Karube et al. (77), who found FOXP3 expression in patients affected by adult T-cell leukaemia/lymphoma (ATLL), an immunosuppressive T cell malignancy characterised by a CD4+CD25+ immunophenotype (65).

In order to investigate the expression of FOXP3 protein in lymphomas we have analyzed a large set of B and T lymphomas using our FOXP3 236A/E7 mAb, which is able to recognise FOXP3 protein in paraffin embedded tissues samples. In agreement with Karube's observation, our results showed that FOXP3 expression in malignant lymphoma cells is restricted to a subpopulation of patients with ATLL, while in the

other lymphoma types analysed, FOXP3 expression was only detected in the reactive T cell population present in the tumour microenvironment.

ATLL is a lymphoproliferative disorder caused by human T-cell lymphotropic virus type-1 (HTLV-1) infection, which is highly endemic, especially in southern Japan and the Caribbean basin. In general, ATLL shows rapid progression, drug resistance and poor prognosis (78). The diversity in the prognosis of patients makes ATLL an extremely complex type of disease, and the existence of a FOXP3 positive ATLL population confirmed the heterogeneity of ATLL in terms of pathological, biological and clinical futures. We were interested in figuring out whether the expression of FOXP3 could be associated with the clinical course of the diseases. To address this question we performed correlation studies between FOXP3 expression and overall survival in ATLL patients, finding that FOXP3+ ATLL may present a more aggressive clinical course, although the difference was not statistically significant. From these findings, we hypothesized that FOXP3+ ATLL could modulate the immune response against the tumour, and could be directly implicated in the generation of the immunodeficiency state commonly associated with ATLL patients (79).

The expression of FOXP3 and its correlation with clinicopathological course was further investigated by Karube et al. in a larger series of patients (80). This new study confirmed that FOXP3 expression was confined to a subgroup of FOXP3+ ATLL, and no differences in overall survival determined by FOXP3 expression were found. This work also demonstrated that FOXP3 expression in ATLL correlated with certain morphological features (including chromosome abnormalities and proliferation of EBV-positive cells), and concluded that the presence of FOXP3 is associated with patients presenting an immunosuppressed clinical state (80).

Although the mechanisms generating the immunodeficiency in ATLL remain unknown, we could speculate that one of the processes implicated in the immunosuppresion state could be the presence of FOXP3 protein in ATLL tumour cells increasing the expression of immunoregulatory molecules on their surfaces, and by the production of immunosuppressive cytokines able to suppress effectors T cells.

On the other hand, the possible mechanisms implicated in ATLL origin are still unclear. Two possible mechanisms have been proposed. As a first hypothesis, HTLV-1 virus could directly target T_{regs}, and changes in FOXP3 expression could be a result of tumour

transformation. Alternatively, leukemic cells could originate after infection of CD4+CD25- T cells, and they could subsequently acquire a T_{reg}-like phenotype. Support for the latter hypothesis is provided by recent data demonstrating that HTLV-1 infection of CD4+CD25- T cells from healthy donors induces a Treg phenotype by up regulating CD25 protein, FOXP3 expression and TGF-β secretion (81).

In conclusion, while FOXP3 expression in ATLL may not predict survival, a significant correlation can be found with the pathological immunodeficient state, illustrating that FOXP3 positive ATLL may function as Treg-like type of tumour. The use of the FOXP3 mAbs generated in this work in the routine diagnostic laboratories could help the identification of ATLL cases worldwide, since FOXP3 expression in tumour cells seems to be restricted to this particular tumour type.

1.3 FOXP3 expression in haematological malignancies and tumour microenvironment

The tumour microenvironment is essential for tumour cell proliferation, angiogenesis, invasion, and metastasis by providing survival signals and an appropriate environment for tumour cells. The microenvironment specific cell composition seems to play an essential role in this scenario, harbouring the ability to develop successful immune responses or, on the contrary, leading to immune evasion and even promoting tumour growth (82).

The cellular composition of the tumour microenvironment, particularly the amount of tumour infiltrating T_{regs}, can significantly mediate cancer immunity. The first report indicating the presence of CD4+CD25+ T_{regs} in human tumour microenvironment emerged in 2001, when Woo et al. reported an increased percentage of T_{regs} in lung and ovarian cancer (83, 84). Since then there has been an explosion of literature describing the presence of T_{regs} in cancer and its correlation with the clinical outcome. Therefore, in the majority of patients afflicted with solid tumours (such as stomach, lung, and ovary cancers), high levels of tumour-infiltrating T_{regs} were associated with a poor prognosis (85), whereas in haematological malignancies, tumour-infiltrating T_{regs} have been demonstrated to have different influences on the clinical outcome.

Thus, in a study run in collaboration with Drs. Piris and Alvaro, the use of our FOXP3 236A/E7 mAb as allowed to assess the relevance of T_{regs} and cytotoxic T lymphocytes

populations (defined by TIA-1 and Granzyme B) in the reactive background of Hodgkin's lymphoma (HL) and its correlation with patient outcome. This study concluded that low infiltration of FOXP3+ cells in combination with high infiltration of TIA-1+ cells represent an independent prognostic factor negatively affecting the survival in this type of lymphoma (see Appendix 1). Furthermore, when the disease relapses and progresses, a larger number of TIA-1+ cells and a lower proportion of FOXP3+ cells are present in the reactive background of the tumour.

A similar result was obtained in a second collaborative study with Dr. Carreras, who reported that follicular lymphoma patients (FL) with higher FOXP3+ T_{regs} numbers in their tumours showed a better response to therapy and improved overall survival (see Appendix 2). This work also described that the number of T_{regs} seemed to be constant in patients who maintained the same cytological grade, but the number was dramatically reduced where the tumour transformed into a diffuse large B cell lymphoma, a more aggressive type of disease (see Appendix 2).

In conclusion, in contrast with most studies in solid tumours, an increased number of T_{regs} seems to be associated with prolonged survival in HL and FL, suggesting that FOXP3+ cells reflect a different biologic aspect of T_{regs} in this type of malignancies. These findings suggest that T_{regs} could promote or constrain tumour cell development depending of the distribution and activation status of the different cells subpopulation present in the tumour microenvironment.

We could hypothesize that in those lymphomas that are invaded by a large inflammatory population, the tumour cells could receive stimulatory signals directly from surrounding inflammatory cells via secreted cytokines and by cell-cell signalling, thus promoting tumour growth and survival (86). We may speculate that in these cases the presence of a high number of regulatory T cells could modulate the interrelationship between tumour and inflammatory cells, hampering tumour cell development. Another possible scenario may be that T_{regs} might have a direct effect on B cells, probably suppressing B cell proliferation by inducing cell death via a cytotoxic-dependent pathway (30, 87, 88).

In any case, all these data are indicative of our incomplete knowledge about the exact mechanisms and the role played by T_{reg} cells in the complex pathophysiology and immune surveillance of lymphoid malignancies. The FOXP3 mAbs generated and

characterized during this work could help in elucidating the mechanisms by which T_{regs} accumulate and function in the tumour microenvironment, as well as in the identification of attractive therapeutic targets potentially useful to fight against tumour-induced immune suppression. Moreover, in case of HL and FL, the detection of a high number of FOXP3+ cells by using these antibodies, is already being applied in the clinical setting as a reliable prognostic marker able to predict improved survival.

2. Discussion second Project

2.1 PD-1 and SAP in Angioimmunoblastic T cell Lymphoma

Angioimmunoblastic T-cell lymphoma (AITL) is a rare and complex lymphoproliferative disorder associated with deregulation of B-cells within a unique malignant microenvironment (89). Non-neoplastic cells represent the major component of ATLL and are composed by a polymorphic inflammatory infiltrate containing small lymphocytes, large lymphoid blasts and follicular dendritic cells, as well as an increased vascular proliferation (90).

AITL usually has a very aggressive course, probably determined by the deregulated immune/inflammatory response rather than by a direct complication of the tumour itself (91). Since the same morphological features can be seen in other conditions, such as in atypical reactive processes, Hodgkin lymphoma, B-cell lymphomas, and other peripheral T-cell lymphoma, the diagnosis of this type of lymphoma remains quite a challenge.

Recent advances in the study of AITL are coming from gene expression profiling studies carried out on peripheral T-cell lymphomas, which have identified T_{FH} as the putative cell of origin of AILT and PD-1 and CXCL13 as the best markers for these T cells subsets (44, 92).

A further relevant molecule that appears to be suitable for identifying T_{FH} cells is the signaling lymphocytic activation molecule-associated protein (SAP), which has been described to be extremely important for T_{FH} physiology, being indispensable for T_{FH} cell signaling and critical for B-T conjugates formation (93). The expression of PD-1 and SAP was not been investigated in human lymphomas due to the lack of antibodies reacting with formalin-fixation resistant epitopes. In an effort to better define the expression and the diagnostic value of PD-1 and SAP molecules in lymphomas we have generated a specific mAb (clone name NAT105) able to recognize PD-1 protein in paraffin embedded tissue, and we have evaluated its expression in a large set of reactive and tumour samples. A commercially available antibody against SAP molecule was also evaluated in this study.

Initially, we found that PD-1 expression was restricted to lymphoid cells and in particular to T cells located in the apex of the light zone in germinal centers of

secondary B-cell follicles. Also, a proportion of lymphocytes in the interfollicular area expressed lower amounts of PD-1.

We confirmed that PD-1 and SAP are co-expressed in the same cell type in the GC, although the presence of scattered SAP+PD1- cells in the interfollicular areas was also detected. This finding suggests that SAP could be induced slightly in advance of PD-1, maybe before T_{FH} enter in the GC.

Among T cell lymphomas, SAP and PD-1 were expressed by the neoplastic cells in the majority of cases of AITL. These data confirm the previous reports that proposed that this disease arise from T_{FH} cells (94, 95).

We reported that SAP and PD-1 are expressed in a minority of T cell lymphomas other than ATLL and in a minority of cases of mycosis fungoides, a cutaneous neoplasm that do not originate from T_{FH} cells. Further studies are needed to redefine those cases of PTCL that lack the classical futures of AITL but do also express SAP and PD-1, raising the question of a possible relationship between these lymphoma types.

We also described that SAP/PD-1 were not expressed in the majority of B cell neoplasms, with the exception of a few cases of DLBCL, where their presence could be due to an occasionally upregulation of these genes in non-B cells, as a result of the neoplastic transformation. SAP expression was also found in more than two thirds of acute lymphoblastic leukemia of T cell origin, in concordance with our observation that cortical thymocytes contain this protein.

In conclusion, our observations provide additional support from the idea that AITL derives from T_{FH} cells, since a large majority of them express SAP and PD-1 molecules.

We also believe that neoplastic transformation of T_{FH} may contribute to the immunodeficiency and immune dysfunction commonly seen in AITL. The defective T cell response could be partially explained by the secretion of TFG-β and IL-10 by T_{FH} resulting in the inhibition of the proliferation and function of conventional CD4 T cells, and consequently resulting in a defective T cell response. In any case, the complex network established among the tumors cells and the various component of the reactive tumor microenvironment is still not well understood. We believe that T_{FH} may play a major role in modulating the growth and survival of neoplastic cells, since this cell type is involved in the recruitment, activation and differentiation of B cells, as well as in modulating other T cell subsets and in promoting vascular proliferation (5, 96).

In summary, PD-1 in combination with CXCL13, ICOS and Bcl6 represents the most useful and robust immunohistochemical T_{FH} marker able to identify this cell subtypes in several experimental settings. Also, PD-1 is extremely useful in the diagnosis of AITL, helping to differentiate this condition from lymphoid hyperplasia and PTCL.

2.2 PD-1 as a tool for the differential diagnosis of nodular lymphocyte-predominant Hodgkin lymphoma

Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is a disease entity distinct from classical Hodgkin lymphoma (cHL), were neoplastic cells (referred as LP cells) are B cells of germinal centre origin usually distributed in large nodular structures of small B cells, scattered histiocytes in association with a network of follicular dendritic cells (FDCs) (97), (98).Furthermore, this type of lymphoma is characterized by an increased number of germinal center-derived CD57+ T cells, which are closely associated with LP cells, often forming rosettes around them. These cells also express markers present in T_{FH} cells such as BCL6, IRF4/MUM1 and c-MAF (99), suggesting that these reactive cells could be T_{FH} cells.

The differential diagnosis between NLPHL can constitute a real challenge, since this type of lymphomas shares several morphological and immunophenotypic similarities with lymphocyte-rich classical Hodgkin lymphoma (LRCHL) and T cell /histiocyte-rich B cell lymphoma (T/HRBCL).

Due to the complexity of the differential diagnosis of NLPHL we assessed the diagnostic and prognostic potential of several T_{FH} markers (CD10, BCL6, CXCL13, CD57 and PD-1) for the identification of T-cell rosettes, since their presence have been proposed as a diagnostic feature of NLPHL.

In our study we found that PD-1-positive and CD57-positive rosettes were present in 98% and 76% of NPHL cases respectively, showing that PD-1 is an effective and sensitive marker for this disease. Noticeably, these results confirm that in this malignancy, tumor cells reside and proliferate in close association with a cellular environment that retains the key features of a normal GC cellular microenvironment.

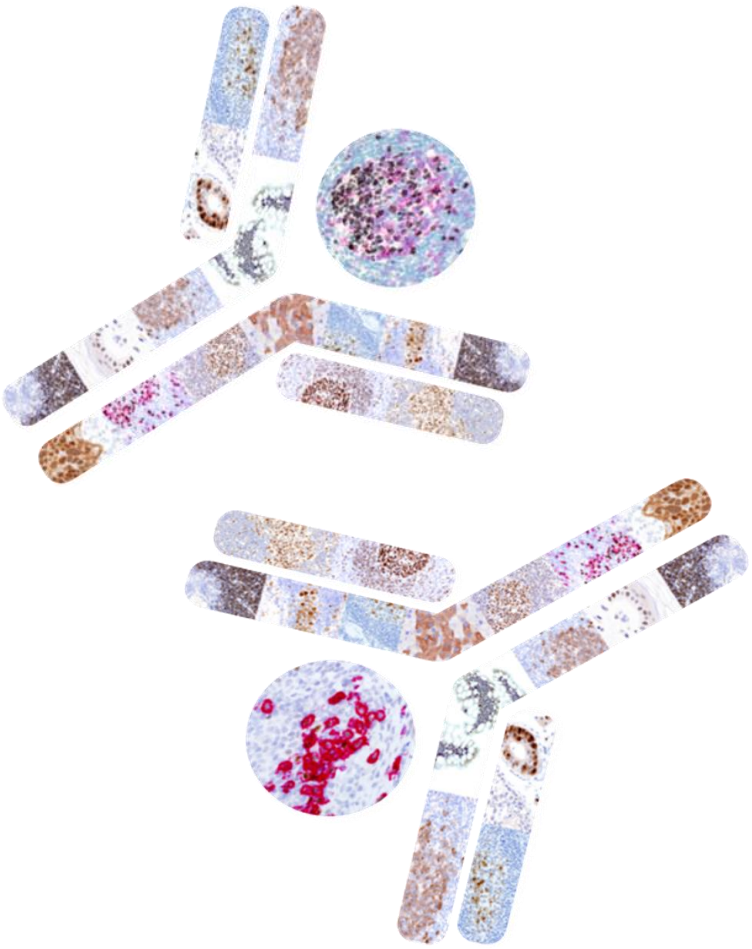
Interestingly, we describe for the first time that the presence of PD-1 positive rosette was not a unique feature of NPHL, but we also found it in several cases of LRCHL. LRCHL is the subtype of cHL the most difficult to differentiate from NLPHL, and

misclassification of this type of tumor has been reported (100). The presence of PD-1 positive rosettes suggests that LRCHL could be a distinct subtype of cHL with characteristics common to both types of lymphomas (101). Furthermore, the atypical tumors cells in both lymphomas share common characteristics, such as overexpression of B cell transcription program markers, and the presence of a follicular T cell background, suggesting a close biological relationship between these entities.

In any case, the most crucial need of a differential diagnosis of NLPHL is with respect to T/HRBCL, since the latter represents a clearly distinct clinicopathologic entity with a more aggressive course, and requiring a different therapeutic management. Both T/HRBCL and NLPHL contain a reduced number of tumour cells that display features of germinal center B cells, but they differ notably in the non-neoplastic microenvironment (102).

In our study, none of the cases that had been diagnosed as T/HRBCL displayed rosettes stained either with CD57 or with PD-1 mAbs, whereas the borderline cases between the two lymphoma types seemed to have follicular T-cell rosettes, thus more closely corresponding with the characteristics of the NLPHL diagnosis group.

We also reported the superior diagnostic value of PD-1 in comparison with CD57, BCL6 and CXCL13. In most types of lymphoma in which T-cell rosettes were present (LRCHL, NLPHL vs. T/HRBCL), PD-1 mAb showed improved staining results, leading to the conclusion that PD-1 should be used as a routine immunomarker especially for the diagnosis of NLPHL and LRCHL.



CONCLUSIONS/CONCLUSIONES

Conclusion first project

1. We have identified and documented for the first time the expression of FOXP3 protein at single cell level in both lymphoid tissue and peripheral blood, showing that FOXP3 is as a specific marker of normal and tumoral T_{reg}.

2. The use of our panel of FOXP3 mAbs allows the characterisation of human FOXP3 protein in a variety of commonly used immunological techniques; in particular its application for FACS analysis has opened new avenues in the investigation of T_{reg} biology and function.

3. FOXP3 expression in malignant lymphoma cells is restricted to a subpopulation of patients with ATLL, while in the other lymphoma types studied, FOXP3 expression was only detected in the reactive T cell population present in the tumour microenvironment.

Conclusion second project

1. We have identified and documented the expression of PD-1 and SAP protein at single cell level in lymphoid tissue, showing that PD-1 and SAP mAbs are specific markers of normal and tumoral T_{FH} cells.

2. PD-1/SAP expression in malignant lymphomas showed that the majority of AITL cases expressed at least one of these markers, providing additional evidence that AITL arises from T_{FH} and that PD-1 is likely to be an extremely valuable tool in the diagnosis of this disease.

3. PD-1 is an excellent marker for the identification of the characteristic T-cell rosettes surrounding neoplastic B-cells in nodular lymphocyte-predominant Hodgkin lymphoma (NPHL).

4. The presence of PD-1 positive rosettes is not a unique feature of NPHL, since we also found this in several cases of lymphocyte-rich classical Hodgkin lymphoma (LRCHL), suggesting a close biological relationship between these entities.

5. PD-1 has an additional useful application in the differential diagnosis of NLPHL, since it can help to discriminate between NPHL and T/HRBCL, which lacks the presence of T cell rosettes.

Conclusiones del primer proyecto

1. Hemos descrito y documentado por primera vez la expresión de la proteína FOXP3 a nivel celular, tanto en tejido linfoide como en sangre periférica.

2. El empleo de nuestro panel de anticuerpos contra FOXP3 permite el estudio de esta proteína mediante una gran variedad de técnicas inmunológicas. En particular, su aplicación en citometría de flujo ha abierto nuevas vías en la investigación de la biología de los linfocitos T_{reg}.

3. La expresión de FOXP3 en linfocitos tumorales se encuentra restringida a una subpoblación de pacientes con ATLL, a diferencia de los otros tipos de linfomas estudiados, donde la expresión tan sólo fue detectada en una población de células T reactivas presentes en el microambiente tumoral.

Conclusiones del segundo proyecto

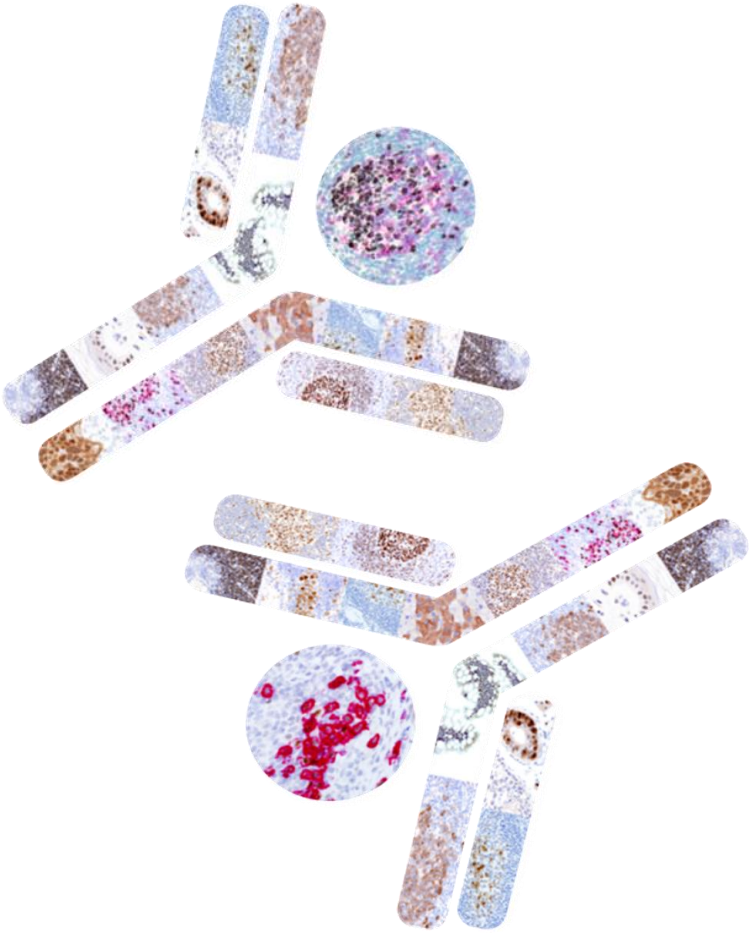
1. Hemos descrito y documentado por primera vez la expresión de las proteínas PD-1 y SAP a nivel celular en tejido linfoide, demostrando que ambos marcadores son específicos de las células T_{FH} normales y tumorales.

2. El estudio de la expresión de PD-1/SAP en linfomas ha mostrado que la mayoría de los casos de AITL expresan al menos uno de estos marcadores, dato que refuerza la hipótesis de que los AITL derivan de las células $T_{FH.}$ PD-1 es por tanto una herramienta muy valiosa en el diagnóstico de esta enfermedad.

3. El anticuerpo generado contra PD-1 es un excelente marcador de las rosetas linfocitarias T que rodean las B neoplásicas, característica clave en el diagnóstico del linfoma de Hodgkin de predominio linfocítico (NPHL).

4. Por primera vez hemos descrito que la presencia de rosetas PD-1 positivas no es una característica exclusiva del NPHL, sino que también se puede encontrar en un porcentaje elevado de linfomas de Hodgkin clásico rico en linfocitos (LRCHL), lo que sugeriría una estrecha relación biológica entre ambas entidades.

5. PD-1 tiene un papel adicional para el diagnóstico diferencial entre NPHL y T/HRBCL, al carecer este último de las características rosetas.



Publications

Publications

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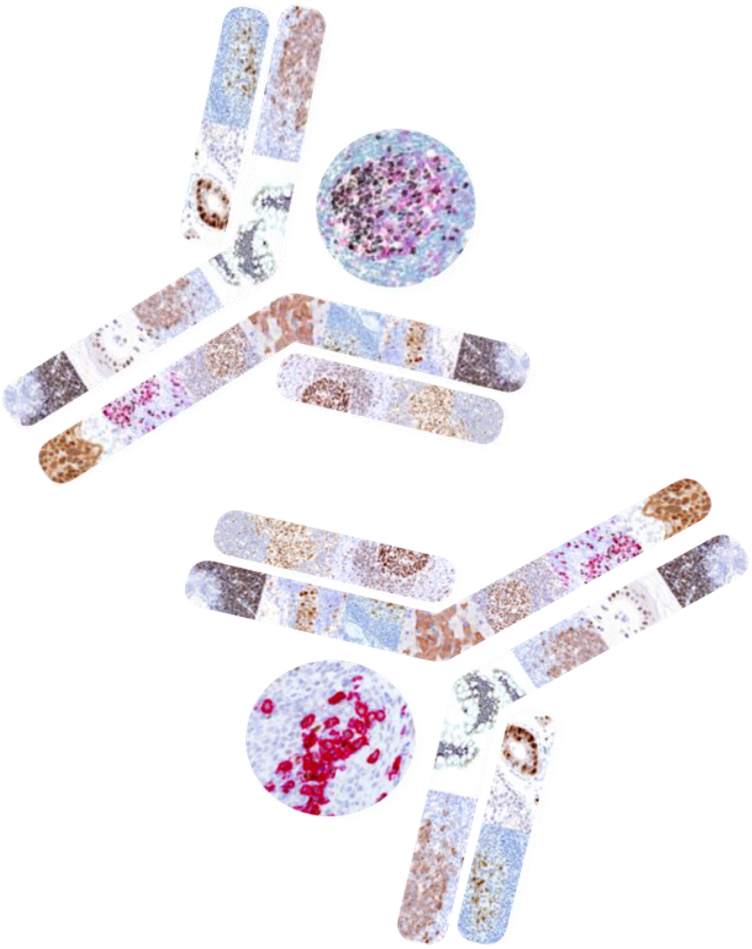

APPENDIX